The Intestinal Microbiome and the Stressed Gastrointestinal Mucosa

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This thesis presents work carried out by myself and does not incorporate without acknowledgment, any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

John Terrence Bresland.

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GIT – Gastrointestinal tract

AGE – Acute gastroenteritis

HBoV2 – Human boca virus 2

RV - Rotavirus

SCFA – Short chain fatty acids

IBS – Irritable bowel syndrome

- D - Diarrhoea dominant IBS
- C - Constipation dominant IBS
- A - Alternating diarrhoea and constipation
- PI - Post infectious IBS

IBD – Inflammatory bowel disease

UC – Ulcerative colitis

CD – Crohn's disease

TJ – Tight junctions

SBBO – Small bowel bacterial overgrowth

PCR –Polymerase chain reaction

qPCR – Quantitative PCR

CH_4 – Methane

H_2 – Hydrogen

H_2S – Hydrogen sulphide

SRB – Sulphate reducing bacteria

L/R ratio – Lactulose/Rhamnose ratio

FC – Faecal calprotectin

IL – Interleukin

TLR – Toll like receptor

NSP 4 – Non-structural protein 4

NFκB – Nuclear factor kappa B

TNF-α – Tumour necrosis factor alpha

Foxp3 – Fork head family transcription factor 3

This thesis is presented in a publication style format, with a general introduction and chapters structured in a manuscript format, containing an introduction, aims, materials and methods, results and discussion section. The references related to each chapter have been compiled at the end of the thesis. This publication style format was selected as this thesis contains content from multiple studies.

Chapter 1 provides background on the intestinal microbiome and its relationship with the stressed gastrointestinal mucosa with the review to be submitted for publication in the Scandinavian Journal of Gastroenterology. This chapter also introduces the notion of a "stressed mucosa" as a site where the interrelationship between the microbiome and the intestinal mucosa are a possible site of disease initiation and a possible target for identifying indicators of mucosal stress.

Chapter 2, has been submitted for publication to the journal of Cancer Biology and Therapeutics. This study shows a model of extreme mucosal stress where depletion in beneficial microbes and an increase in potentially harmful bacteria enhance mucosal damage during chemotherapy. The data suggests that manipulation of the microbiome may help in ameliorating symptoms of chemotherapy-induced mucositis.

Chapter 3 is also prepared as a manuscript to be submitted for publication in the journal of Journal of Pediatric Gastroenterology and Clinical Nutrition. This study shows that levels of endogenous *Bifidobacterium* spp. were related to diarrhoeal severity in children with rotavirus infection. The data also shows that two different viral infections differentially alter the intestinal microbiome compared to healthy aged matched control subjects.

Chapter 4 is to be submitted for publication in the journal of Gut Pathogens. This study in a cohort of IBS patients where a degree of mucosal stress is assumed shows, several relationships between non-invasive markers of mucosal stress and endogenous bacteria of the intestinal microbiome.

Chapter 5 is to be submitted for publication in the Journal of Breath Research. This study investigates the relationship between microorganisms controlling the redox potential and fermentative activity in the colon with non-invasive markers of mucosal stress in a well-classified cohort of IBS patients. This study has shown that levels of H_2 and CH_4 produced in the colon may have a role in decreasing intestinal permeability and inflammation in certain IBS classifications.

The general discussion links these disparate studies together under the banner of a stressed mucosa and discusses the use of non-invasive methods of evaluating the compositional shifts in the microbiome and the gastrointestinal mucosa under stress. This chapter also presents a hypothetical ratio of a known pathogenic species to beneficial bacteria as a possible index of mucosal stress. These findings suggest a link between endogenous members of the microbiome and diarrhoeal severity and that the fermentative patterns of the microbiome may influence mucosal patency. These findings now set the scene for future trials to investigate the role of the intestinal microbiome and non-invasive methods of analysis of the aforementioned and the relationship with the gastrointestinal mucosa under stress in a number of different disease settings.

Stress has been defined as any event that modifies the capability of an organism to maintain homeostasis. A stressed mucosa can be induced by iatrogenic causes, including chemotherapy and non-steroidal anti-inflammatory drugs (NSAIDs), infectious agents, such as viral and bacterial pathogens, or it can be idiopathic, such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Collectively, clinical events related to a stressed mucosa are associated with enormous health and societal burdens worth billions of dollars in direct health care costs with related loss of productivity.

The most important findings of this thesis are related to the composition and activity of the microbiome, which differs between diseases and syndromes characterized by mucosal damage and mucosal stress. Relationships within the endogenous microbial composition have been shown in this thesis, and between endogenous microbial composition and non-invasive biomarkers of a damaged, stressed or inflamed mucosa. These relationships may significantly influence host response and the severity, progression and duration of disease. Relatively few studies have investigated the balance of the faecal microbiome and its association with indicators of mucosal dysfunction in gastrointestinal diseases of differing severity.

The three selected examples of mucosal damage and stress of differing severities used in this thesis are a rat model of chemotherapy-induced mucositis, paediatric rotavirus (RV) and human bocavirus 2 (HBoV2) infections, and IBS. The compositional balance of the microbiome with respect to known and putative probiotic/beneficial species were quantitated using PCR. These were compared to candidate pathogens, such as *E. coli,* in these disparate settings.

In the rat model of chemotherapy-induced mucositis, changes affecting the composition of the microbiome were shown to be associated with the degree of mucosal injury and severity of diarrhoea. Specifically, significantly reduced levels of the mucosally protective endogenous *Bifidobacterium* and *Lactobacillus* spp. with a concomitant increase of *E. coli* and sulphate reducing bacteria (SRB) were associated with the severity of both mucosal damage and diarrhoea.

In paediatric patients with RV (known aetiology) and HBoV2 (newly discovered) associated diarrhoea, alterations to composition of the microbiome were observed between these two different viral infections compared to healthy age matched controls. A significant correlation was observed between endogenous levels of *Bifidobacterium* spp. and frequency of diarrhoea in RV infection. Non-invasive detection of microbiome changes during childhood are important to understand, because of the related sequelae that sometimes follow infection.

There are currently no markers for diagnosis of IBS therefore non-invasive indicators of microbiome changes and mucosal integrity may provide potential management strategies for the clinician. This study reports for the first time significant correlations between endogenous bacteria, non-invasive markers of mucosal stress and intestinal gases (H_2 and CH_4) in a cohort of IBS patients. Levels of *Bifidobacterium* spp. appeared to protect from diarrhoea in patients with IBS-D. Furthermore, levels of H_2 appeared to promote a more patent small intestinal mucosa, and levels of CH_4 correlated with decreased inflammation in the gastrointestinal tract (GIT).

In summary, this thesis comprises studies, which have shown that endogenous beneficial bacteria, markers of intestinal permeability and inflammation and intestinal gases may be

related to the degree of mucosal damage in multiple mucosal stress situations. This thesis describes changes in the microbiome during stress events, providing some scientific evidence to support future intervention studies in a range of clinical settings that selectively augment the indigenous microbiome populations with either probiotics, prebiotics or synbiotics to promote not only mucosal health, but also the maintenance and restoration of the mucosal barrier under stress.

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Finally, I would like to thank my wonderful partner Tara Ellen for indulging me with the time, space and opportunity to complete this enormous task. It truly is a long strange ride my love and I thank you for all your encouragement, patience, never ending cups of tea and your Buddha like qualities over the course of this endeavour. Now, let us build a straw bale house together!!! To my three beautiful children, Ned, Alice and little Audrey, I would like to say thank you for being patient over the last couple of years, I'm very lucky indeed to have you all in my life, you've all been wonderful, I love you dearly.

John 2015.

Chapter 1.0 The intestinal microbiome and the stressed gastrointestinal mucosa

1.1 Introduction

The intestinal microbiome is a consortium of trillions of microorganisms coexisting together, with a balanced correctly functioning microbiome positively associated with gut health and human wellbeing (Clemente, Ursell, Parfrey *et al*. 2012). However, compositional shifts in the microbiome have been associated with a range of illnesses and syndromes (Tannock 2008, Salonen, De Vos and Palva 2010, Shanahan 2012). The intestinal mucosa is the largest interface in contact with the outside environment, and is exquisitely sensitive to changes in physiological parameters responsible for the maintenance of homeostasis. Stress has been defined as any event that modifies the capability of an organism to maintain homeostasis (Davidson, Kritas and Butler 2007). A stressed mucosa can be induced by iatrogenic causes (including chemotherapy and non-steroidal anti-inflammatory drugs (NSAIDs) (Wardill and Bowen 2013), infectious agents (such as viral and bacterial pathogens), or it can be idiopathic (such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)) (Codling, O'mahony, Shanahan *et al.* 2010). Collectively, clinical events related to stressed mucosa are associated with enormous health and societal burdens worth billions of dollars in direct health care costs with related loss of productivity (Chey, Kurlander and Eswaran 2015).

The intestinal microbiome is a complex assemblage of microorganisms with a vast genetic and metabolic diversity (Lozupone, Stombaugh, Gordon *et al*. 2012, Aziz, Doré, Emmanuel *et al*. 2013). Interactions between the mucosa and the intestinal microbiome are complex and are positively correlated with human health (Clemente, Ursell, Parfrey *et al*. 2012). Under conditions of stress, mucosal function can be compromised leading to a breakdown in the mucosal barrier. This can result in a more permeable intestinal mucosa that permits

luminal antigens, including microorganisms and their metabolites to interact with immune cells, resulting in a heightened immune response (Figure 1.1) (Davidson, Kritas and Butler 2007). Emerging evidence suggests that compositional shifts in the intestinal microbiome can predispose individuals to a dysregulated immune response and chronic physiological reactions. However, relatively few studies have comprehensively correlated the microbial population with clinical parameters associated with a stressed mucosa. Although the aetiology and pathogenesis of mucosal stress is not clearly defined, it has been hypothesised that stress events in any form (physical or psychological) can alter how the intestinal microbiome and mucosa communicate, driving the pathophysiology of stressed mucosa.

Figure 1.1. Stressed mucosa.

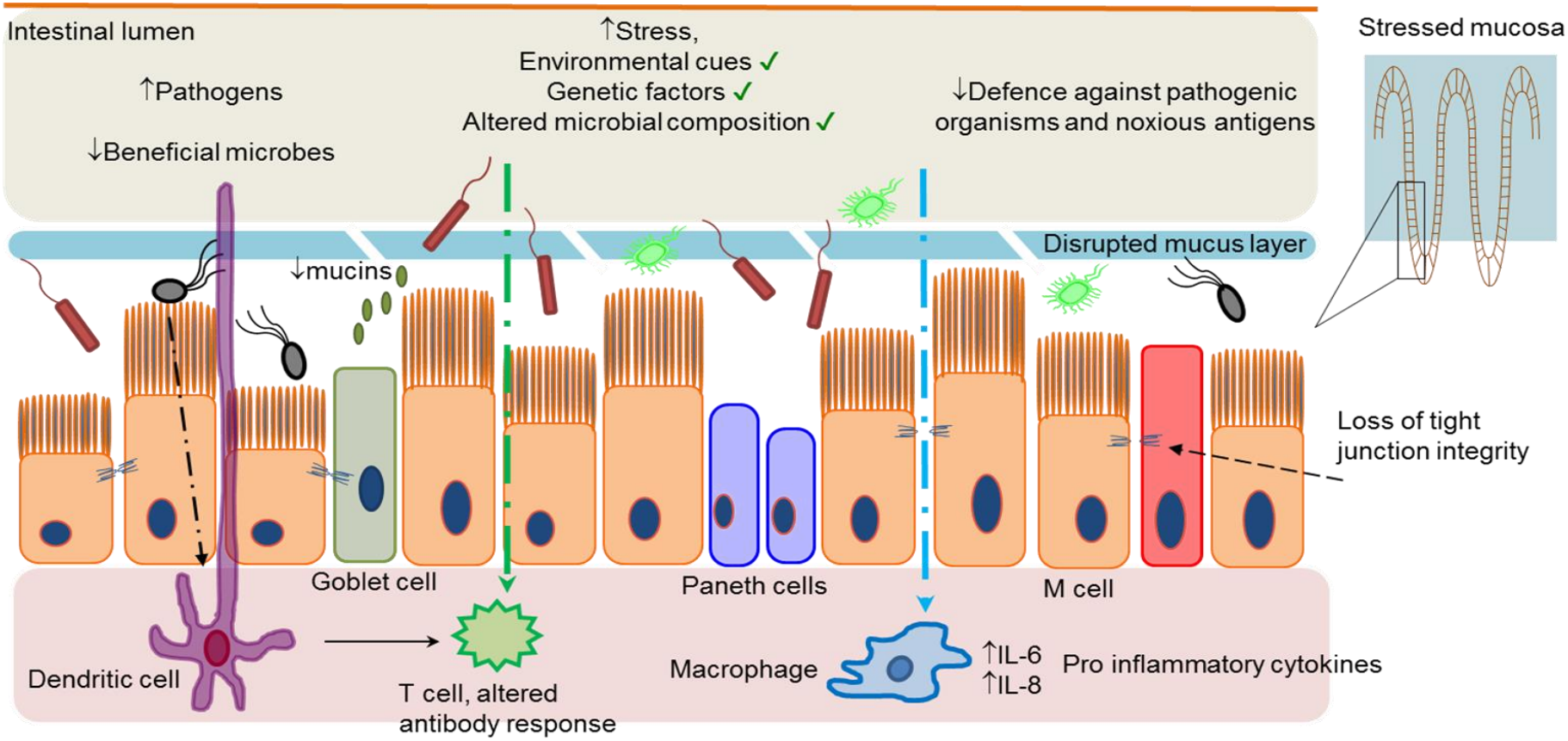

Figure 1.1. Stressed mucosa. Loss of tight junction integrity allowing access of bacteria, pathogens, antigens and bacterial products to interact with immune cells embedded in the lamina propria triggering an immune response. The mucus layer may become thin, decreasing its physical role in preventing access to the underlying tissue. Decreased mucus may also play a role in impaired barrier function as it offers less adhesion sites for beneficial bacteria to out compete pathogenic bacteria.

1.2 Iatrogenic causes of a stressed mucosa

1.2.1 Chemotherapy-induced mucositis

Mucositis is a debilitating and potentially life threatening complication occurring, to some degree, in up to 100% of patients who receive cancer chemotherapy or radiotherapy (Bowen and Keefe 2008). The risk of mucositis has been directly associated with treatment modality, intensity and route of delivery of the cancer therapy. However, it is increasingly apparent that additional risk factors, such as genetic polymorphisms, compositional changes in the intestinal microbiome, and altered mucin expression may predispose patients to a higher severity of mucositis (Bowen, Gibson, Tsykin et al. 2007, Stringer, Gibson, Logan *et al*. 2009).

Mucositis presents with symptoms from the oral cavity (pain and erythema, dysphagia and food aversion) to the intestine (abdominal pain, diarrhoea, ulceration and bleeding) (Sonis, Elting, Keefe et al. 2004). Moreover, translocation of pathogens through the damaged intestinal barrier may lead to septicaemia, with catastrophic consequences (Sonis, Elting, Keefe *et al*. 2004). These devastating symptoms can lead to reductions in chemotherapy dose and duration, decreasing the probability of successful tumour killing and remission (Keefe 2006). This has a significant impact on the patient's quality of life, as well as placing greater pressure on the Australian health care system, with the clinical management of mucositis requiring costly multiple adjuncts to alleviate pain, and to reduce symptomology.

The pathogenesis of intestinal mucositis is complex and is determined by a number of interacting factors (Sonis, Elting, Keefe *et al.* 2004). The initiating steps are characterised

by an increase in apoptosis, which is followed by a reduction in crypt length and villus height (Keefe, Brealey, Goland *et al.* 2000). This is accompanied by an increase in the production of reactive oxygen species, leading to significant tissue damage. Up-regulation and generation of different transcription factors, nuclear factor kappa B (NF-κB) is pivotal amongst these (Sonis 2002), and leads to the activation of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF)-α, interleukin (IL)-1β, and IL-6 (Logan, Stringer, Bowen *et al*. 2007). These pro-inflammatory cytokines have direct damaging effects on the mucosal cells and activate a cascade and amplification of different pro-inflammatory pathways that can lead to further tissue injury. During the ulcerative phase of mucositis, there is a robust inflammatory infiltrate, which is comprised of polymorphonuclear cells, macrophages and lymphocytes, leading to further cytokine amplification and inflammation (Logan, Gibson, Bowen *et al.* 2008). This may lead to a weakening of the intestinal barrier, facilitating the migration of harmful pathogens and leading to further complications, including sepsis. The healing phase is characterised by a rebound hyperplasia of the intestinal crypts and villi and recovery of intestinal function (Al-Dasooqi, Gibson, Bowen *et al.* 2010).

1.2.2 Non-steroidal anti-inflammatory drugs and mucosal stress.

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most widely used treatments for inflammatory conditions worldwide, with approximately 30 million people ingesting them regularly (Kamil, Geier, Butler *et al.* 2007). Commonly used NSAIDs include indomethacin, aspirin, naproxen, ibuprofen and ketoprofen, which all act by binding to cyclooxygenase isozymes. Despite their therapeutic efficacy, long term use of NSAIDs is associated with gastrointestinal side-effects and a stressed mucosa (Kamil, Geier, Butler *et*

al. 2007). NSAID induced stressed mucosa manifests primarily as ulceration, leading to bleeding, increased intestinal permeability and inflammation, which can be life threatening with a 10% mortality rate in patients admitted to hospital with NSAID related bleeding (Singh and Triadafilopoulos 2005). The toxicity of NSAIDs limits their safe use in a range of settings such as elderly patients, and patients with a predisposing gastrointestinal illness (Singh and Triadafilopoulos 2005).

1.3 Infectious causes of mucosal stress

1.3.1 Viral causes of mucosal stress

1.3.1.1 Rotavirus

Acute gastroenteritis (AGE) is an inflammatory condition of the GIT caused by viral or bacterial pathogens leading to severe diarrhoea (Arthur, Higgins, Davidson *et al.* 2009). AGE is a life threatening illness causing mortality and morbidity in children worldwide (Arthur, Higgins, Davidson *et al.* 2009). Until the early 1970's a causative agent responsible for AGE in paediatric populations with severe diarrhoea was largely unknown. Indeed, only 20% of cases were identified as having a defined causative agent.

In 1973, Bishop *et al*, identified rotavirus (RV) as the primary causative agent for acute paediatric gastroenteritis (Bishop, Davidson, Holmes *et al.* 1973). RV has since been found to be the most common cause of AGE worldwide in children under 5 years of age accounting for up to 50% of all cases (Bishop 2009). RV is a naked double stranded RNA virus transmitted by the faecal oral route, with five defined serotypes (A to E) with A being the most common to infect humans (Lundgren and Svensson 2001, Hyser and Estes 2009). The incidence and related mortality of RV has decreased in developed nations due to the

introduction of vaccines, however RV infection is still responsible for over 500,000 deaths annually in children under the age five in the developing world (Kirkwood, Boniface, Bishop *et al.* 2009).

The pathophysiology of RV is multifactorial, with diarrhoea caused by direct mucosal damage to the small intestinal mucosa. RV damages the apical cells lining the duodenum and jejunum, causing necrosis, which leads to malabsorption and profuse watery diarrhoea. The secretion of non-structural protein (NSP 4) also disrupts tight junctions between intestinal epithelial cells (Ramig 2004). In a polarized epithelial cell line (MDCK-1), NSP 4 leads to disruption of the tight junctional complexes between intestinal epithelial cells, and increased paracellular permeability (Tafazoli, Zeng, Estes *et al.* 2001). Additionally, NSP 4 stimulates the enteric nervous system and influences the secretion of water into the lumen leading to severe diarrhoea (Ramig 2004).

The development and licensing of two RV vaccines RotaTeq (Merck and Co, PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium) has resulted in decreased cases in developed countries (Tate, Burton, Boschi-Pinto *et al.* 2012). However, despite the introduction in the developed world, RV is still a major cause of mortality and morbidity in the developing world with more research needed into this debilitating and life threatening illness (Tate, Burton, Boschi-Pinto *et al*. 2012).

1.3.1.2 Norovirus

Norovirus is highly infectious illness acquired from food and spread through the faecal oral route and human-to-human contact (Donaldson, Lindesmith, Lobue *et al.* 2008). Norovirus causes severe gastroenteritis in confined human populations such as hospitals, military barracks and cruise ships, resulting in 267,000,000 annual infections (Donaldson, Lindesmith, Lobue *et al.* 2008). Moreover, norovirus accounts for over half of all gastroenteritis outbreaks worldwide (Atmar and Estes 2006). Unlike RV, norovirus affects all ages, the immunocompromised and the elderly (Glass, Parashar and Estes 2009). Norovirus is associated with mild to severe gastroenteritis, with vomiting and diarrhoea of short duration (1-3 days) (Wilhelmi, Roman and Sanchez-Fauquier 2003). Hospitalisation occurs for severe cases, which are predominantly confined to the elderly. Norovirus is associated with damage to the small intestine (duodenum and jejunum), causing intestinal villous blunting, crypt-cell hyperplasia, and infiltration of polymorphonuclear and mononuclear cells into the lamina propria (Glass, Parashar and Estes 2009). Interestingly, commensal bacteria may enhance norovirus infection. Antibiotic treated mice with a depleted intestinal microbiome had decreased titers of murine norovirus compared with non-antibiotic treated mice, suggesting that the microbiota may play an active role in norovirus infection (Jones, Watanabe, Zhu *et al.* 2014).

1.3.1.3 Astrovirus

Astrovirus is associated with diarrhoea in children under two years of age, but can affect immunocompromised adults and the elderly (Wilhelmi, Roman and Sanchez-Fauquier 2003). Astrovirus may account for up to 20% of sporadic non-bacterial diarrhoeas and up to 15% of diarrhoeal outbreaks in Australia (Kirkwood, Clark, Bogdanovic- Sakran *et al.* 2005). Astrovirus is a mild illness rarely leading to dehydration, with symptoms lasting 3–4 days

(Wilhelmi, Roman and Sanchez-Fauquier 2003). A unique feature of astrovirus is that the capsid appears to act like an enterotoxin, and disrupts tight junction complexes leading to increased intestinal permeability and is independent from replication (Moser, Carter and Schultz-Cherry 2007).

1.3.1.4 Human boca virus 2

The newly discovered human boca virus 2 (HBoV2) is a parvovirus and was recently associated with AGE in an Australian paediatric population (Arthur, Higgins, Davidson *et al.* 2009). HBov2 may represent up to 15% of undiagnosed causes of AGE in children (Arthur, Higgins, Davidson *et al.* 2009). HBoV2 has recently also been identified in an American study involving adults and children and was associated with gastrointestinal symptoms (Chow, Ou and Esper 2010). Currently there is no data on the possible causative mechanism of HBoV2 infection.

The sequelae of illnesses that result from acute viral infections of the GIT have not been fully explored. However, there is emerging evidence suggesting that abrupt changes in the intestinal environment that disrupt either barrier function or intestinal permeability can stress the mucosa (Bischoff, Barbara, Buurman *et al.* 2014). Interactions of the microbiota and the stressed mucosa following acute viral events are poorly understood. Research is needed to characterise population shifts occurring in the microbiome during acute viral infection, which may aid the development of targeted protocols, aimed at restoring the microbial ecosystem to promote health.

1.3.2 Bacterial causes of mucosal stress

Bacterial gastroenteritis can alter the intestinal microbiome and predispose a proportion of patients to a range of illnesses, including increasing susceptibility to IBS (Spiller and Campbell 2006). There is little information regarding the changes to the microbiota and the mucosa during both bacterial and viral infections of the GIT, and the implications post infection. A meta-analysis of studies investigating IBS by Halvorson *et al* (2006), found a 7 fold increase in the risk of developing IBS post infectious gastroenteritis (Halvorson, Schlett and Riddle 2006). Of the 8 studies included in the analysis, four failed to report a pathogen responsible for the gastroenteritis, while the remaining four reported known bacterial pathogens, such as Salmonella and Shigella (Halvorson, Schlett and Riddle 2006). In an 8-year follow up study, Thabane *et al* (2010), also reported an increased incidence of IBS in both adults and children, following an outbreak of AGE caused by *E. coli* O157:H7 and *Campylobacter jejuni* (Thabane, Simunovic, Akhtar-Danesh *et al.* 2010). Mechanisms for the increased incidence of IBS post infection remain poorly understood. One possible mechanism for this phenomenon is the inability of the mucosa-microbiome co-mutualism to return to the pre infective state. Thus the infection may disrupt barrier function, alter the commensal microbial balance and the cross talk between the mucosa and the microbiota creating a chronic inflammation that drives IBS symptoms (Thabane, Simunovic, Akhtar-Danesh *et al.* 2010).

1.3.2.1 Escherichia coli

E. coli is one of the main microorganisms causing diarrhoea in people world-wide, and transmission has been associated with poor hygiene/sanitation and poor food preparation (Majowicz, Scallan, Jones-Bitton *et al.* 2014). *E. coli* is a commensal microbe in the GIT of humans. However, particular strains such as O157;H7 can result in life threatening illnesses

such as, haemorrhagic colitis and haemolytic uraemic syndrome (Dibaise, Zhang, Crowell *et al.* 2008, Schüller 2011). *In vitro*, enteropathogenic *E. coli* causes disruption to tight junctions between epithelial cells by increased myosin light chain phosphorylation resulting in increased intestinal permeability (Yuhan, Koutsouris, Savkovic *et al*. 1997). Other stains of *E. coli,* such as O157;H7 produce effector proteins EspF and EspFU that disrupt host cells, leading the mucosal remodelling and increased intestinal permeability (Schüller 2011). *E. coli* is a common pathogen encountered worldwide leading to severe gastroenteritis, which can cause intestinal permeability and mucosal stress.

1.3.2.2 *Campylobacter* spp.

Campylobacter spp., including *C. jejuni*, *C. coli*, and *C. fetus*, are a common source of food borne gastroenteritis worldwide (Acheson and Allos 2001, Man 2011). *C. jejuni* is one of the most commonly encountered food borne bacterial pathogens, leading to severe gastroenteritis in humans. Mucosal abnormalities have been reported in animal models with chickens showing decreased villous height and crypt depth, accompanied by increased intestinal permeability in the caecum (Awad, Molnár, Aschenbach *et al.* 2015). In other animal models, *C. jejuni* infection causes increased translocation of intestinal pathogens across the mucosal barrier by disrupting tight junction proteins such as occludin, resulting in increased interactions of luminal microorganisms and their metabolites with immune cells embedded in the lamina propria resulting in intestinal inflammation (Chen, Ge, Fox *et al.* 2006).

C. jejuni affects the GIT in humans by attaching to intestinal epithelial cells and secreting a number of toxins that directly disrupt barrier function, leading to increased intestinal permeability (Man 2011). Increased permeability across the mucosal barrier facilitates the movement of lipo-oligosaccharides on the outer cell wall of *C. jejuni* to interact with T cells in affected nerve cells, triggering Guillain Barre Syndrome. Guillain Barre Syndrome is a debilitating autoimmune disorder that leads to motor impairment and paralysis (Ang, Jacobs and Laman 2004). Despite being the most common form of food poisoning, little is understood about the chronic effects of *C. jejuni* infection and the long-term effects on the mucosa after the infection has resolved.

1.3.2.3 *Salmonella* spp.

Non-typhoid salmonella poisoning is another common infectious disease causing AGE resulting in fever, vomiting and severe diarrhoea (Santos, Tsolis, Bäumler *et al.* 2003). *Salmonella* spp. ingestion via contaminated food or water causes salmonella poisoning. *Salmonella* spp. cause a mucosal stress that is characterised by neutrophil infiltration to the apical region of the intestinal epithelium, triggered by the induction of IL-8 (Mccormick, Colgan, Delp-Archer *et al.* 1993). *Salmonella enterica* serovar *typhimurium* also affects intestinal permeability by targeting Rho GTPase, which alters the formation of the actinonmycin ring involved in tight junctions (Boyle, Brown and Finlay 2006). Salmonellosis is usually a self-limiting illness resolving 1–3 days after onset of symptoms. However, a dysregulated immune response following infection can predispose those infected to polyarthritic diseases such as reactive arthritis and Reiter's syndrome (Dworkin, Shoemaker, Goldoft *et al.* 2001, Tuompo, Hannu, Mattila *et al.* 2013). This has been shown in a population following a confirmed outbreak of gastroenteritis caused by *S. enterica*,

where 29% of patients with confirmed salmonella poisoning were diagnosed with reactive arthritis and Reiter's syndrome following the outbreak. Patients with severe diarrhoea greater than 7 days duration (and theoretically increased mucosal stress) that required hospitalization were at higher risk of developing reactive arthritis and Reiter's syndrome (Dworkin, Shoemaker, Goldoft *et al*. 2001).

Recently, Cremon *et al* 2014, reported an increased incidence of functional dyspepsia and IBS following a salmonella outbreak in Italy (Cremon, Stanghellini, Pallotti *et al*. 2014). In this long term prospective study (16 years following infection) analysis of the outbreak found that children were more susceptible to IBS symptoms as adults when compared to adults who were unexposed as children (Cremon, Stanghellini, Pallotti *et al.* 2014). This study combined with others is compelling circumstantial evidence that supports the hypothesis that disruptions to the microbiota are possibly linked with the onset of IBS symptoms. Indeed, acute gastroenteritis from any cause will undoubtedly lead to a stressed mucosa and a dysregulated mucosal immune response elicited by altered microbial environment in the GIT.

1.4 Idiopathic causes of mucosal stress

1.4.1 Inflammatory bowel disease

IBD is a debilitating chronic inflammatory T cell mediated disease with relapsing periods of inflammation and high life time morbidity where stressed mucosa is clearly evident (Kalischuk and Buret 2010). IBD is characterised by defined sub-types: ulcerative colitis (UC), which is localised only to the colon; and Crohn's disease (CD), which can manifest along the entire length of the gastrointestinal tract (Louis, Dotan, Ghosh *et al*. 2015). The aetiology of IBD is a complex interplay of host genetic factors, environmental cues and the intestinal microbiome (Bouma and Strober 2003). IBD is characterised by a heightened immune response leading to severe mucosal inflammation, which is evident by both biopsy and raised faecal calprotectin levels, which spike during active disease (Sartor 1995).

Histologically, the mucosa in CD patients is altered, showing transmural inflammation with aggregates of macrophages in the epithelial layer (granulomas). Conversely, in UC patients reoccurring crypt abscesses lead to changes in crypt architecture (Geboes 2003), increased polymorphonuclear leukocytes, and decreased goblet cells leading to mucosal stress. The chronic ulceration and inflammation of the mucosal lining observed in IBD patients severely compromises the ability of the mucosa to separate the luminal environment from the mucosal immune system. Emerging evidence is implicating the intestinal microbiome as a critical initiating factor in the pathogenesis of IBD. Microbial diversity is significantly decreased in IBD patients, characterised by amended levels of Clostridial group IV (*Faecalibacterium prausnitzii*) (Frank, Amand, Feldman *et al.* 2007, Sartor 2010). *F. prausnitzii* has been shown to be anti-inflammatory, with increased levels observed in healthy controls compared to patients with IBD (Frank, Amand, Feldman *et al.* 2007, Sokol,

Seksik, Furet *et al.* 2009). Animal studies have demonstrated the importance of microbial shifts in priming the intestinal mucosa to a pro-inflammatory state. Normal, wild-type mice receiving a faecal transplant from mice with colitis and metabolic syndrome show increased susceptibility to developing colitis and have altered metabolism (Garrett, Lord, Punit *et al.* 2007, Vijay-Kumar, Aitken, Carvalho *et al.* 2010). Faecal transplant has been proposed as a clinical strategy to shift the microbiome of IBD patients to a non-pathogenic state. Faecal transplant is now an accepted treatment modality for patients with *C. difficile* infection, and recent clinical trials have suggested that the same could be applied to IBD. By developing a greater understanding of the intestinal microbiome, and its compositional shifts during mucosal stress, more targeted approaches may be used to restore microbial balance.

1.5 Functional gastrointestinal disorders

Functional gastrointestinal diseases (FGID) are a cluster of common gastrointestinal disorders characterised by recurring and persistent gastrointestinal symptoms (Drossman 2006, Chang, Locke, Mcnally *et al.* 2010). There are no structural (tumours or masses) or biochemical abnormalities present. However, they occur as a consequence of abnormal gastrointestinal function (Drossman 2006, Chang, Locke, Mcnally *et al.* 2010). There are over 20 FGID identified, affecting all parts of the GIT including oesophagus, small intestine, colon, stomach and biliary regions (Chang, Locke, Mcnally *et al.* 2010). FGID are an increasing economic burden on the health care resources of industrialised countries and although mortality is not related to FGID, they significantly impact quality of life (Chang, Locke, Mcnally *et al.* 2010). Of all the FGID, irritable bowel syndrome (IBS) is the most widely studied and most common. The estimated economic burden of IBS in 2015 directly and indirectly in the USA is proposed to be $20 billion (Chey, Kurlander and Eswaran 2015).

1.5.1 Irritable bowel syndrome

IBS affects between 9-21% of the world's population (Palframan, Gibson and Rastall 2003, Halvorson, Schlett and Riddle 2006, Spiller 2007, Salonen, De Vos and Palva 2010), with prevalence increasing in the developed world (Gwee 2005). IBS is defined by abdominal pain and discomfort that occurs in conjunction with altered bowel habits for a period of at least three months (Brandt, Chey, Foxx-Orenstein *et al.* 2009). There is an increased incidence among females compared to males of 2:1 (Schneider 2013). IBS affects all age groups (Canavan, West and Card 2014), and is the most common ailment to be seen by gastroenterologists (Broe 2013). The Rome criterion was developed as a diagnostic aid for the classification of FGID. Rome 111 divides IBS into four distinct phenotypes: IBS-D (diarrhoea), IBS-C (constipation), IBS-A (alternating constipation and diarrhoea) and IBS-PI (irritable bowel syndrome that was preceded by infectious gastroenteritis) (Salonen, De Vos and Palva 2010).

There is still no clearly defined aetiology for the occurrence of IBS (Hungin, Becher, Cayley *et al.* 2015). Some of the proposed mechanisms involve low grade chronic inflammation, changes in the intestinal microbiome, increased intestinal permeability, psychosocial factors, dysmotility, genetic factors, bile acid malabsorption, changes in brain morphology and dietary triggers (Codling, O'mahony, Shanahan *et al.* 2010).

1.5.2 The microbiome and irritable bowel syndrome

Compositional changes in the microbiome of IBS patients are largely undefined; however, an increasing body of evidence is implicating altered microbial composition in IBS compared to healthy patients (Table 1.1). Alterations in the predominant beneficial bacteria appear to

be accompanied by rises in potentially harmful microorganisms, which may promote an altered luminal environment affecting the interactions between the mucosa and the intestinal microbiome. The mucosally beneficial bacterium *F. prausnitzii* is consistently lower in faeces of IBS patients compared with healthy control levels (Rajilić–Stojanović, Biagi, Heilig *et al*. 2011) (Table 1.1). Also, lower levels of endogenous *Bifidobacterium* spp. which bolster barrier function and are associated with a healthy GIT are consistently lower in faeces when compared to healthy controls (Kassinen, Krogius-Kurikka, Mäkivuokko *et al.* 2007) (Table 1.1). Levels of the mucosally protective *Lactobacillus* spp. were almost undetectable in the clone libraries of IBS patients. Levels of another bacterium associated with a healthy intestinal tract *Collinsella aerofaciens* were also found to be lower in the IBS patients when compared to healthy controls (Kassinen, Krogius-Kurikka, Mäkivuokko *et al.* 2007). These studies demonstrate alterations in the intestinal microbiome of IBS patients and show decreased microbial diversity and loss of beneficial microorganisms. Although these differences do not directly suggest causation, they do indicate that the microbiome is altered in IBS patients when compared to the microbiome of healthy controls.

Table 1-1: Summary of molecular and culture based studies of colonic microbiome in IBS.

Subject	Sampling method	Method	Findings	Study
IBS (n=25 Controls (n=25)	Faeces (adult)	Culture	↓*Bifidobacterium* spp. ↓*Clostridium perfringens* ↑Enterobacteriaceae	China, (Si, Yu, Fan *et al.* 2004)
IBS (n=20 Controls (n=20)	Faeces (adult)	Microarray	↓*Bifidobacterium* spp. ↓Bacteroidetes ↑Proteobacteria ↑Firmicutes	Finland, *(*Rajilic-Stojanovic, Smidt and De Vos 2007*)*
IBS (n=24) Controls (n=23)	Faeces (adult)	qPCR, Microarray	↓*Lactobacillus* spp. ↓*Collinsella aerofaciens*	Finland, (Kassinen, Krogius-Kurikka, Mäkivuokko *et al.* 2007)
IBS (n=11) UC (n=13) Controls (n=22)	Faeces (adult)	PCR-DGGE	↓Bacterial diversity ↓*Bacteroides* spp.	UK, (Noor, Ridgway, Scovell *et al*. 2010)
IBS (n=47) Controls (n=33)	Faecal Colonic biopsies (adult)	PCR-density gradient gel electrophoresis (DGGE)	No difference between faeces and biopsy	Ireland, *(*Codling, O'mahony, Shanahan *et al.* 2010*)*

Table 1.1: continued

Subject	Sampling method	Method	Findings	Study
IBS (n=11) Controls (n=11)	Faeces (children)	454 Pyrosequencing	↑Gammaproteobacteria ↑*Veillonella* spp.	USA, (Saulnier, Riehle, Mistretta *et al*. 2011)
IBS (n=62) Controls (n=46)	Faeces (adult)	qPCR, Global and deep molecular analysis	↓*Bifidobacterium* spp. ↓*Faecalibacterium prausnitzii* ↓Bacteroidetes ↑Firmicutes:Bacteroidetes	Finland, (Rajilić–Stojanović, Biagi, Heilig *et al.* 2011)
IBS (n=37) Controls (n=20)	Faeces (adult)	Pyrosequencing	↓Bacteroidetes ↑Firmicutes:Bacteroidetes ↓Actinobacteria	Sweden, (Jeffery, O'toole, Öhman *et al.* 2012)
IBS (n=47) Controls (n=30)	Faeces (adult)	qPCR	↓*Bifidobacterium* spp. ↑*Pseudomonas aeruginosa* ↓*Bacteroides* spp.	India, (Shukla, Ghoshal, Dhole *et al.* 2015)
IBS (n=21) Controls (n=74)	Duodenal aspirate (adult)	qPCR/culture	↑*Escherichia/Shigella* ↑*Klebsiella pneumoniae*	USA, (Giamarellos-Bourboulis, Tang, Pyleris *et al*. 2015)

1.5.3 IBS phenotypes and the microbiome

Specific microbial profiles have been associated with the different IBS phenotypes (Table 1.2). Using molecular based techniques Krogius-Kurikka *et al* 2009, showed decreased levels of mucosally beneficial *Bifidobacterium* spp. and the large commensal group *Bacteroides* spp. in IBS-D patients when compared to healthy controls, with a concomitant rise in *E. coli* and the Firmicutes phyla (Krogius-Kurikka, Lyra, Malinen *et al.* 2009). In other studies differences between the microbiota of IBS-PI and general IBS patients, and controls have been reported. Sundin *et al* 2015, reported faecal and mucosal microbes tend towards the higher levels of the Bacteroidetes in IBS-PI and cluster towards the *Clostridium* cluster XIVa and IV in general IBS patients without an episodic prior infection (Sundin, Rangel, Fuentes *et al.* 2015). Decreased faecal and mucosal microbial diversity in IBS-PI was also reported when compared to healthy controls. Increased sensitivity to visceral pain is a hallmark symptom of IBS. Butyrate is a known beneficial short chain fatty acid (SCFA) and is associated with a healthy colonic mucosa and may be related to visceral pain perception (Vanhoutvin, Troost, Kilkens *et al.* 2009). *F. prausnitzii* a major butyrate producer within the GIT was lower in IBS patients when compared to controls. Increased levels of *Ruminococcus bromi* and *R. gnavus* were also seen in IBS patients compared to controls. *Ruminococcus* spp. are acetate and H_2 producers which may lead to excess gas production and be associated with symptoms in both IBS-PI and general IBS (Sundin, Rangel, Fuentes *et al.* 2015).

Table 1-2: Summary of molecular studies comparing colonic microbiome differences between phenotypes of IBS and healthy controls.

Subject	Sampling method	Method	Patient group	Findings	Study
IBS (n=20) Controls (n=15)	Faeces	qPCR	IBS-D IBS-C IBS-A	↑*R. torques* ↑*Clostridium thermosuccinogenes* ↑*R. bromi* ↑*C. thermosuccinogenes* ↑*R. torques*	Finland, (Lyra, Rinttilä, Nikkilä *et al*. 2009)
IBS (n=12) Controls (n=22)	Faeces	%G+C profiling	IBS-D	↓*Bifidobacterium* spp. ↓*Lactobacillus* spp ↑Proteobacteria ↑Firmicutes	Finland, (Krogius-Kurikka, Lyra, Malinen *et al*. 2009)
IBS (n=10) Controls (n=10)	Faecal, Colonic mucosal biopsy	qPCR, Culture	IBS-D	↓Aerobic bacteria ↑*Lactobacillus* spp.	USA, (Carroll, Chang, Park *et al*. 2010)
IBS (n=23) Controls (n=23)	Faeces	454 high throughput sequencing	IBS-D	↑Enterobacteriaceae ↓*F. prausnitzii* ↓Diversity	USA, (Carroll, Ringel-Kulka, Siddle *et al*. 2012)

Table 1.2: continued

Subject	Sampling method	Method	Patient group	Findings	Study
IBS (n=47) Controls (n=30)	Faeces	qPCR	IBS-D	↑*Bacteroides thetaiotaomicron* ↑Segmented filamentous bacteria ↓*Lactobacillus* spp.	India, (Shukla, Ghoshal, Dhole *et al*. 2015)
			IBS-C	↑*Lactobacillus* spp. ↓Segmented filamentous bacteria	
IBS (n=32 Controls (n=16)	Faeces Mucosal samples	Phylogenetic microarray hybridisation	IBS-PI	↓Microbial diversity	Sweden, (Sundin, Rangel, Fuentes *et al.* 2015)
			IBS	↑*R. gnavus* ↑*R. bromi* ↓*F. prausnitzii*	

1.5.4 Inflammation in irritable bowel syndrome

IBS has been characterised as a chronic low-grade inflammatory syndrome with no clearly distinct precursors (Hungin, Becher, Cayley *et al.* 2015). Low-level activation of innate and adaptive mucosal immune responses has been previously reported in IBS patients (Chadwick, Chen, Shu *et al.* 2002, Öhman and Simrén 2010, Barbara, Cremon, Carini *et al*. 2011). Mast cells are associated with hypersensitivity in the GIT and release a number of molecules including histamine and tryptase. Both histamine and tryptase are responsible for the recruitment of inflammatory cells into the gastrointestinal mucosa. The amount and extent of inflammation in IBS is still yet to be systematically shown. However, emerging evidence indicates that inflammation is present in IBS and that certain categories of IBS may have a more inflammatory profile than others (Ford and Talley 2011).

1.5.5 Intestinal permeability and irritable bowel syndrome

There is growing body of evidence that suggests disrupted intestinal permeability may play a causative role in IBS symptomatology (Martínez, Lobo, Pigrau *et al.* 2013). The dual sugar test is a marker of small intestinal permeability, and was shown to be high in over 50% of IBS suffers compared to healthy controls (Piche, Barbara, Aubert *et al.* 2009). Rao *et al* 2011, found that intestinal permeability was altered between IBS patients, healthy controls and a group of ulcerative colitis patients with known diminished barrier function (Rao, Camilleri, Eckert *et al.* 2011). Functional changes in the integrity of the mucosal barrier allow the penetration of luminal contents activating local immune responses (Shen and Turner 2006). This has been recognized as a contributing factor leading to low grade inflammatory response and disruption of the mucosal barrier in IBS (Dunlop, Hebden, Campbell *et al.* 2006, Martínez, Vicario, Ramos *et al.* 2012). In a clinical assessment of IBS-D patients

jejunal biopsies were analysed and compared to healthy controls. This study found that the distribution of the tight junction proteins was altered leading to enlarged apical intercellular spaces (Martínez, Lobo, Pigrau *et al.* 2013). These changes in cellular ultrastructure correlated with clinical symptoms and with mast cell activation compared to healthy controls (Martínez, Lobo, Pigrau *et al.* 2013). This study demonstrates that there may be an organic cause rather than a functional one in some IBS patients. Furthermore, this study and others highlight the complex nature of IBS, and more importantly indicate that a disrupted mucosal barrier leading to increased permeability is a major site of immune system dysregulation, initiation and progression of IBS (Drossman 2006).

1.6 The microbiome

The intestinal microbiome is an enormous bacterial community outnumbering our own somatic human cells 10 to 1 with a genome 150 times larger than that of the human host (Qin, Li, Cai *et al.* 2012, Lepage, Leclerc, Joossens *et al.* 2013). Acquired at birth and changing throughout infancy, the microbiome stabilises in early adulthood and remains relatively stable throughout life (Faith, Guruge, Charbonneau *et al.* 2013). The microbiome can be viewed as an organ acquired at birth with the metabolic capacity of the liver (Sansonetti 2010). This is further enhanced by the ability of the microbiome to shift gene expression in response to a vast array of diverse glycan's (carbohydrates) ingested as part of the human diet. There are an estimated 1000 bacterial species in each individual intestinal microbiome, with predictions of up to 40 000 species for the whole microbiome (Tap, Mondot, Levenez *et al*. 2009).

The human GIT microbiome consists of microorganisms from a number of different phyla but is dominated by two, the Firmicutes and Bacteroidetes, which make up 90% of all GIT microorganisms, with the former being the largest group (Moeller and Ochman 2014). The remaining microbes such as methanogens make up a cluster of organisms performing specialised functions within the microbiome, such as the removal of hydrogen (H_2) produced by bacterial fermentation in the colon. Methanogens are strict anaerobes which utilize the reducing power H_2 along with the simple carbon compounds produced through fermentation to produce methane (Rudolf 1998). Other phyla represented in the intestinal microbiome are Actinobacteria, Proteobacteria and Fusobacteria, making up 5, 8 and 1%, respectively (Candela, Maccaferri, Turroni *et al.* 2010). The remaining phyla comprise Cyanobacteria, Verrucomicrobia, Euryarchaeota, Lentisphaerae, Synergistetes, Planctomycetes and the Deinococcus-Thermus group (Candela, Maccaferri, Turroni *et al.* 2010, Rajilić- Stojanović and Vos 2014).

The human intestinal microbiome is a dynamic, metabolically active microbial ecosystem playing a number of essential roles in human physiology (Xu and Gordon 2003). The microbiome has the genomic capacity to perform a wide range of metabolic and catabolic functions, which are exploited by the host, including vitamin synthesis (vitamin K, B12, biotin and thiamine). This is exemplified by germ free mice requiring vitamin supplementation (Vitamins K and B including B12, biotin, folic acid, and pantothenate) to maintain health compared with conventionally raised mice with an inhabited intestinal tract (Sumi, Miyakawa, Kanzaki *et al.* 1977, Wostmann 1981). Other functions of the microbiome are processing of xenobiotics, methanogenesis, degradation of complex carbohydrates into smaller mono and disaccharides, production of SCFA's through fermentation in the colon,

maturation of mucosal immunity, offering protection from pathogens, tightening of barrier function by influencing cell proliferation and differentiation and modification of behaviour/mood (Bäckhed, Ley, Sonnenburg *et al.* 2005, Candela, Maccaferri, Turroni *et al.* 2010, Lepage, Leclerc, Joossens *et al*. 2013).

The microbiome is essential to ensure immune system maturation and to promote overall health of the organism. Germ-free rats, raised without a diverse colonized intestinal tract have decreased crypt cell number and reduced number of colonic crypts (Alam, Midtvedt and Uribe 1994). Furthermore, germ free mice are more susceptible to infection than conventionally raised mice, with key parameters such as reduced immune system maturation, decreased numbers of immune cells in the intestinal lamina propria and decreased intraepithelial lymphocytes characteristic of the absence of an intestinal microbiome. (Alam 1995). Furthermore, Peyer's patches of germ free mice are smaller and lack germinal centres compared with mice harbouring a diverse array of microorganisms (Shroff, Meslin and Cebra 1995).

1.6.1 Regions of the gastrointestinal tract

The intestinal tract can be broadly divided into the upper and lower GIT: The upper GIT consists of, the mouth, oesophagus and stomach while the lower GIT consists of the small intestine and colon. Each region can be further separated into smaller ecological niches.

1.6.1.1 Microbiome of the upper gastrointestinal tract

The bacterial diversity of the oral cavity rivals that of colon, with the density being lower (Consortium 2012). There are over 700 species originating from different phyla, including viruses, protozoa, fungi, bacteria and one archaea (Wade 2013). Firmicutes represent the dominant phyla in the oral cavity with *Streptococcus* spp. being the most abundant. The main selective features for bacteria inhabiting the mouth are surface attachment sites such as, cheek, teeth and tongue and the predominantly aerobic environment (Aas, Paster, Stokes *et al.* 2005, Dethlefsen, Eckburg, Bik *et al.* 2006).

1.6.1.2 Oral cavity

The oral microbiome is an important component of oral health and systemic health (Wade 2013). The microorganism of the oral cavity provide mucosal protection from invading microbes by decreasing adhesion sites (Wade 2013). Mucosal stress in the oral cavity can caused by alcohol, drugs and tobacco and can affect mucosal permeability (Ogden 2005). Alcohol has been shown to increase permeability in porcine oral mucosa with the effect dependant on the percentage of alcohol. 50% alcohol increased the passage of large molecules such as albumin (Howie, Trigkas, Cruchley *et al.* 2001). Increased permeability of the oral mucosa may allow the passage of potential carcinogens and contribute to cancers of the oral cavity (Squier, Cox and Hall 1986). Antibiotic treatment can also stress the oral mucosa as is shown by increased susceptibility to opportunistic infections after antibiotic use such candidiasis (Sullivan, Edlund and Nord 2001).

1.6.1.3 The oesophagus

The oesophagus was once thought to have a very minimal microbiome due to the fast transit time of ingesta. Recent studies have identified the microbial composition of the oesophagus to be more complex than previously thought with over 100 species identified (Lawson and Coyle 2010). Indeed, the oesophageal microbiome may be implicated in severe mucosal stress of oesophagitis and Barrett's oesophagus (BE) (Yang, Lu, Nossa *et al.* 2009). Yang *et al* 2009, was able to classify the oesophageal microbiomes into type 1 or type 11, where type 1 was associated with a normal oesophagus (increased *Streptococcus* spp.) and type 11 classified with oesophagitis and BE (had significantly reduced levels of *Streptococcus* spp.) and increased levels of gram-negative anaerobes and microaerophilic bacteria) (Yang, Lu, Nossa *et al.* 2009). Mucosal stress is clearly evident in oesophagitis and BE and suggests that an altered microbial composition may trigger pathological changes leading to stress in the oesophageal mucosa (Yang, Lu, Nossa *et al.* 2009).

1.6.1.4 The stomach

The stomach microbiome consists of bacteria washed down from the oral cavity into the stomach with food and ingested fluid. The stomach is a hostile environment for microbes with many organisms killed upon entering the stomach due to the low pH (around 2). Recently, with the use of non-culture based techniques such as PCR the gastric environment has been shown to have a clearly definable bacterial composition (Macfarlane and Dillon 2007, Yang, Nell and Suerbaum 2013). The finding that *Helicobacter pylori* survives and thrives leading to gastritis (mucosal stress) was the first clue that other bacteria could survive in this harsh environment (Bik, Eckburg, Gill *et al.* 2006). *H. pylori* is a controversial bacterium as it is a causative agent in gastric cancer and on the other hand its

decreased carriage has been associated with increased risk for a range of illnesses including oesophageal cancer, gastroesophageal reflux disease, asthma and allergy (Lin and Koskella 2015). *H. pylori* may well be a commensal microbe that is required for a balanced microbial ecosystem in the stomach. Further studies are needed to understand the role of *H. pylori* in the functioning of the gastric microbiome and more importantly how this organism may be mucosally protective.

The functioning of the stomach has a major influence on the bacterial composition of the microbiome in the colon (Kanno, Matsuki, Oka *et al.* 2009). This occurs when acid secretion is reduced as in patients with chronic atrophic gastritis. In these patients hyposecretion of acid altered levels of *Veillonella* and *Lactobacillus* spp. with levels of *Bifidobacterium* spp. remaining unchanged (Kanno, Matsuki, Oka *et al.* 2009). Although recent findings have indicated that the stomach has a distinct microbiome many events can alter the composition, such as *H. pylori* infection and changing acid secretion which may initiate disease (Maldonado-Contreras, Goldfarb, Godoy-Vitorino *et al.* 2011).

1.6.2 Microbiome of the lower gastrointestinal tract

1.6.2.1 The microbiome of the small intestine

The small intestine is predominately the site of nutrient absorption and assimilation. The density of bacteria in the healthy duodenum and jejunum is in the order of 10^2 bacteria/mL. Low numbers of microorganisms in the upper small intestine (duodenum and jejunum) are due to fast transit times of ingesta (2 to 4 hours), low pH of contents as they exit the stomach, and toxicity of bile salts (Figure 1.2) (Macfarlane and Dillon 2007). Density and diversity of bacteria start to gradually increase along the length of the small intestine, gradually

becoming more anaerobic toward the distal ileum (Figure 1.2) (Xu and Gordon 2003, Macfarlane and Dillon 2007, Sartor 2008, Quigley 2010). The dominant bacteria found in the upper small intestine is *Streptococcus* spp., *Veillonella* spp., and *Lactobacillus* spp. However, very little is known about the small intestinal microbiome as its location creates major problems for sampling (Santos, Tsolis, Bäumler *et al.* 2003).

Figure 1.2. Anatomical diagram of the gastrointestinal tract.

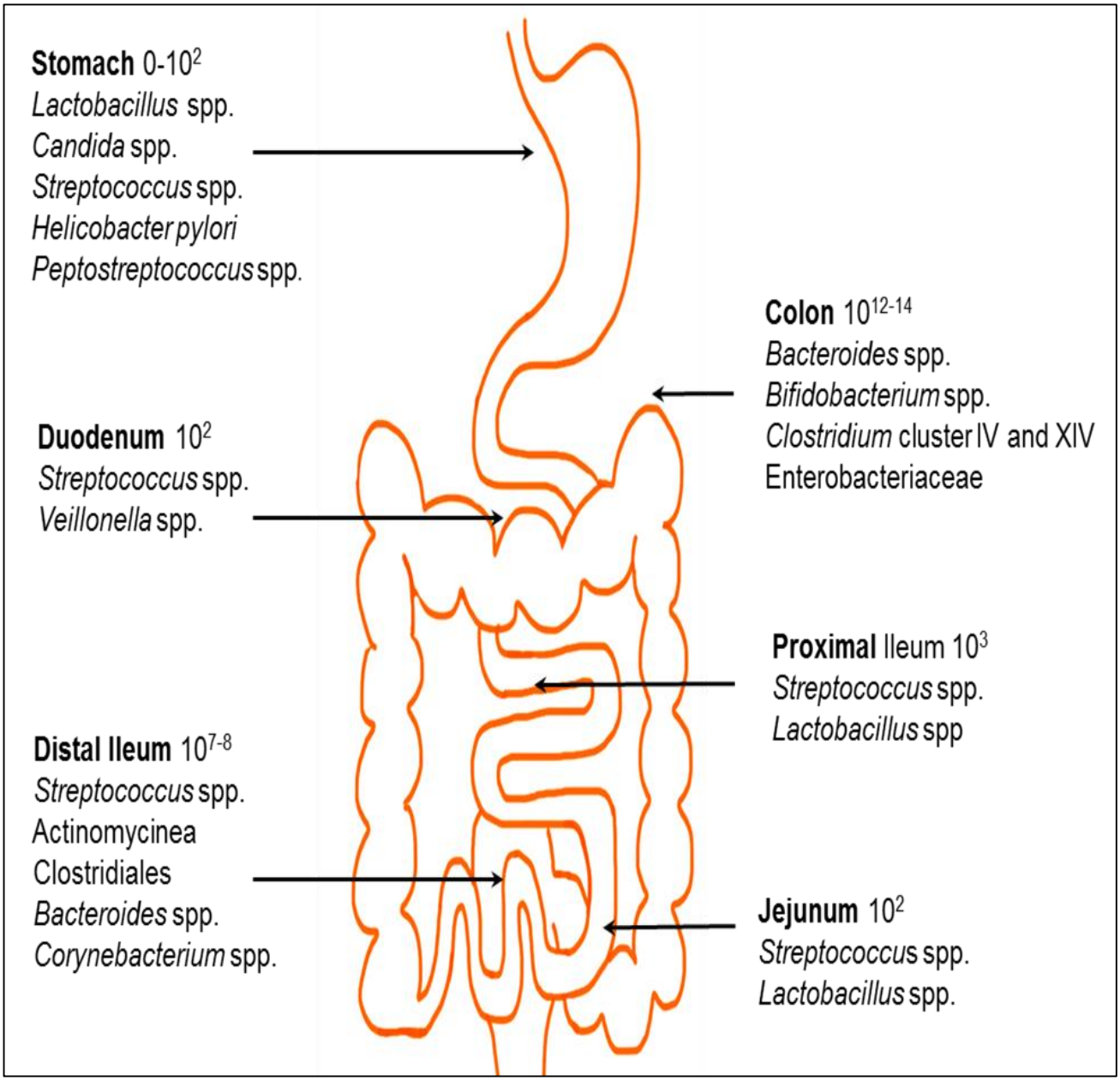

Figure 1.2. Anatomical diagram of the gastrointestinal tract. Relative abundances of bacteria in each geographical niche of the gastrointestinal tract (bacteria/mL luminal contents).

1.6.2.2 The microbiome of the colon

The colon of healthy human adults harbours the highest population of microorganisms within the body (Xu and Gordon 2003). Beginning at the ileocecal valve the GIT becomes anaerobic with the microbiota increasing exponentially in diversity and density (Xu and Gordon 2003). The large intestine is divided into four geographical regions: the cecum, ascending colon, transverse colon and the descending colon, with each region having a distinctive microbiota. Numbers of microorganisms gradually rise from 10^8 bacteria/ml in the caecal contents and luminal contents of the ascending colon, to 10^{14} bacteria/mL in the distal colon and faeces (Dethlefsen, Eckburg, Bik *et al.* 2006). The colon is dominated by obligate anaerobes (Ley, Peterson and Gordon 2006), with the majority of bacteria arising from two dominant phyla, Firmicutes and Bacteroidetes (Samuel, Hansen, Manchester *et al.* 2007). A large proportion of the bacteria that reside in the colon are mostly fermentative microbes, which are suited to the anaerobic environment of the colon. The Firmicutes, (gram positive) are the largest group of microorganisms in the GIT comprising, *Lactobacillus* spp., *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Clostridium* spp., *Ruminococcus* spp., *Veillonella* spp. and *Eubacterium* spp. Bacteroidetes (gram negative) comprise *Bacteroides* and *Prevotella* species (Sjogren, Tomicic, Lundberg *et al.* 2009). The third predominant phylum is the Actinobacteria containing three orders, Actinomycetales, Bifidobacteriales and Coriobacteriales (Kassinen, Krogius-Kurikka, Mäkivuokko *et al.* 2007). There are currently three identified archaea responsible for methanogenesis within the human colon they are, *Methanobrevibacter smithii, Methanosphaera stadtmanae* and *Methanomassiliicoccus luminyensis* (Dridi, Henry, El Khéchine *et al*. 2009, Dridi 2012).

1.6.3 Intestinal gases that define the redox state and fermentation of the microbiome.

1.6.3.1 Hydrogen production

Fermentation of carbohydrates is the only source of H_2 within the human body. The capacity of the microbiome to produce H_2 gas is a process that can be exploited by clinicians to detect bacterial overgrowth in the small intestine, gastrointestinal transit times and lactose malabsorption (Di Camillo, Marinaro, Argnani et al. 2006, Yu, Cheeseman and Vanner 2011). Non-digestible sugar probes are commonly used to investigate parameters of gut function. Specific sugar probes are ingested and breath samples collected and measured to indicate when H_2 production commences. When H_2 is recorded early in the test (less than 90 minutes after ingestion), this indicates bacteria have actively metabolised the test probe and produced H_2 (Tooley, Howarth and Butler 2009). This is an abnormal result, as no fermentation should occur in the small intestine. A positive test result is an indicator of increased levels of fermentative bacteria in the small intestine, which may indicate small bowel bacterial overgrowth (SBBO). This example indicates the usefulness of H_2 production and its role in detecting imbalances in the small intestine (Tooley, Howarth and Butler 2009).

Recently, H_2 gas has been shown to have antioxidant capabilities and reduces damage to the brain and liver through ischemia/reperfusion injury by mopping up hydroxyl radicals (Fukuda, Asoh, Ishikawa *et al.* 2007). Ischemia/reperfusion injury can occur in a variety of ways such as mesenteric artery occlusion and small intestinal transplantation (Schoenberg and Beger 1996) and liver transplant. After surgery when the intestine is perfused, hydroxyl radicals generate pro inflammatory cytokines, which enhance local intestinal injury. Furthermore, it can also affect intestinal barrier function causing increased permeability leading to systemic inflammation and sepsis causing multiple organ failure (Collard and

Gelman 2001). In one study small intestinal transplantation was performed on male rats. The recipient and donor received 2% H_2 plus 98% air (treatment group) or air alone (control group). This studied reported that the addition of H_2 in the treatment group reduced mucosal damage and enhanced barrier function when compared to control group. H_2 gas was also able to decrease transplant-induced suppression of intestinal motility compared to the air treated rats. H_2 was also found to decrease pro-inflammatory cytokine production which was associated with a reduced inflammatory response and reduction in hydroxyl radicals responsible for tissue damage postoperatively (Buchholz, Kaczorowski, Sugimoto *et al*. 2008). The production of H_2 by the intestinal microbiome may act as a moderate mucosal antioxidant and as such contribute to mucosal health.

1.6.3.2 Methanogenesis

Methanogenesis is the result of microbial decomposition of organic substrates in a anaerobic environment (Rudolf 1998). Methanogenic archaea are represented in both vertebrates and invertebrates (Lin and Miller 1998). The production of methane is not catalysed by a single microbe, but is the result of syntrophic relationships between numbers of microorganisms in the GIT. Fermentation of resistant starches in the colon produce a range of products such as, (carbon dioxide (CO_2), H_2), acetate, butyrate, propionate and formate). The production of H_2 through bacterial fermentation in the GIT is a rate-limiting step in the degradation of ingested carbohydrates. The capacity of the microbiome to manage H_2 is referred to as the hydrogen economy.

Methanogens are members of the Archaea phyla and are obligate anaerobes that use H_2 to reduce CO_2 and/or methanol to methane (CH_4), thereby disposing of excess H_2 (Carbonero, Benefiel and Gaskins 2012). Stoichiometrically, methanogenesis converts 4 moles of H_2 to 1 mole of CH_4, thereby drastically reducing the amount of free H_2 in the GIT. Methanogenesis is therefore thought to be essential to prevent the accumulation of H_2 and CO_2 in the colon (Samuel, Hansen, Manchester *et al*. 2007, Dridi, Henry, El Khéchine *et al.* 2009). Methanogenic archaea are one of a number of organisms that control levels of H_2 within the colon (Samuel, Hansen, Manchester *et al.* 2007). The ability of methanogens to utilise H_2 creates a thermodynamically efficient environment for a range of gut microbes to increase energy salvage in the form of acetate, and promote bacterial butyrate production, which is vital as the primary energy source for colonocyte health (Samuel, Hansen, Manchester *et al*. 2007). Archaea and the interaction with fermentative bacteria have been reported in obesity indicating that selected microorganisms of the microbiome become increasingly efficient at harvesting energy in the form of acetate, which may then lead to increased adiposity (Dibaise, Zhang, Crowell *et al.* 2008). Sulphate reducing bacteria can also reduce a number of intestinal metabolites to decrease levels of H_2. Reductive acetogens are the third in the triumvirate to use H_2 to reduce CO_2 to acetate. Breath analysis can detect CH_4 produced by the microbiome. Levels of methanogens required to produce CH_4 on the breath range from 10^{6-8} per gram of faeces (Miller and Wolin 1982). It has recently been shown that 99% of people harbour methanogens, although not all will produce methane detectable on breath.

Methanogens were first identified from human faeces using anaerobic culturing methods and methane detection in breath (Miller and Wolin 1982). *Menthanobrevibacter smithii* has

been identified as the dominant methanogen in the human GIT and was almost ubiquitous in 700 stool samples tested (Dridi, Henry, El Khéchine *et al.* 2009). *Mbb. smithii* is the dominant archaea in the GIT representing up to 10% of all microbes, whereas the methylotrophic *Methanosphaera stadtmanae* is detected less frequently and at lower levels. Recently, a third archaea was isolated from human stool, *Methanomassiliicoccus luminyensis* (Dridi 2012).

It has been reported that for detection of CH_4 in breath, methanogen numbers have to reach between 10^6-10^8/g dry weight of faeces (Weaver, Krause, Miller *et al.* 1986). It has also been shown that not all who harbour methanogens excrete methane in the breath. Indeed, breath methane has only been detected in up to 36% in a US population (Levitt, Furne, Kuskowski *et al*. 2006). However, this varies in different environments, for example >60% of children in Burma expire CH_4 (Mello, Tahan, Melli *et al.* 2012). It has been proposed that the inability to detect CH_4 in the breath could be due to the high intake of protein providing dietary sulphate or alternatively the detection methods lack sufficient sensitivity. Increased dietary sulphate in the western diet may promote the growth of SRB within the colon inhibiting methanogen growth, leading to less than 1 ppm of CH_4 in breath observed in some western populations (Christl, Gibson and Cummings 1992). As yet this conundrum is yet to be resolved.

The role of CH_4 in constipation predominant IBS (IBS-C) is still to be clearly identified (Furnari, Savarino, Bruzzone *et al.* 2012). However, there appears to be a correlation between CH_4 and altered motility in this subset of IBS patients and other constipated

patients (Soares, Lederman, Fagundes-Neto *et al.* 2005, Chatterjee, Park, Low *et al.* 2007). Methanogens have also been detected in anorexic patients, where they may play a role in directing mucin degradation by mucin degrading bacteria thereby increasing acetate production when food intake in disrupted or absent (Armougom, Henry, Vialettes *et al.* 2009). Archaea play a crucial regulatory role in managing H_2 and other products of fermentation within the GIT. The exact role of CH_4 and archaea within the colonic environment and the relationship to health and disease and its potential use as a barometer of colonic health is as yet unresolved.

1.7 Functional roles of the gastrointestinal tract

1.7.1 Barrier function

The definition of barrier function is “a functional entity separating the gut lumen from the inner host, and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensins, IgA), immunological elements (lymphocytes, innate immune cells), muscular and neurological elements” (Bischoff, Barbara, Buurman *et al.* 2014). The intestinal barrier is a protective mechanism against pathogenic bacteria and noxious antigens. These arise from the external environment and from those that may be locally produced, for example, hydrogen sulphide (H_2S), a mucosal irritant produced from sulphate reducing bacteria in the colon. The GIT barrier comprises a range of epithelial cells, each performing different roles (Figure 1.3) (Ulluwishewa, Anderson, Mcnabb *et al.* 2011, Giorgio, Miele, Principessa *et al.* 2014). Barrier function differs throughout the GIT based on local and specific functional requirements such as nutrient absorption and waste secretion (Turner 2009). Disturbances effecting barrier function may expose individuals to luminal and microbial components, initiating a cascade of inflammatory pathways leading to stressed

mucosa. Better understanding of the mechanisms promoting/regulating barrier function will lead to novel intervention strategies, and better diagnostic techniques.

Figure 1.3. Healthy intestinal mucosa.

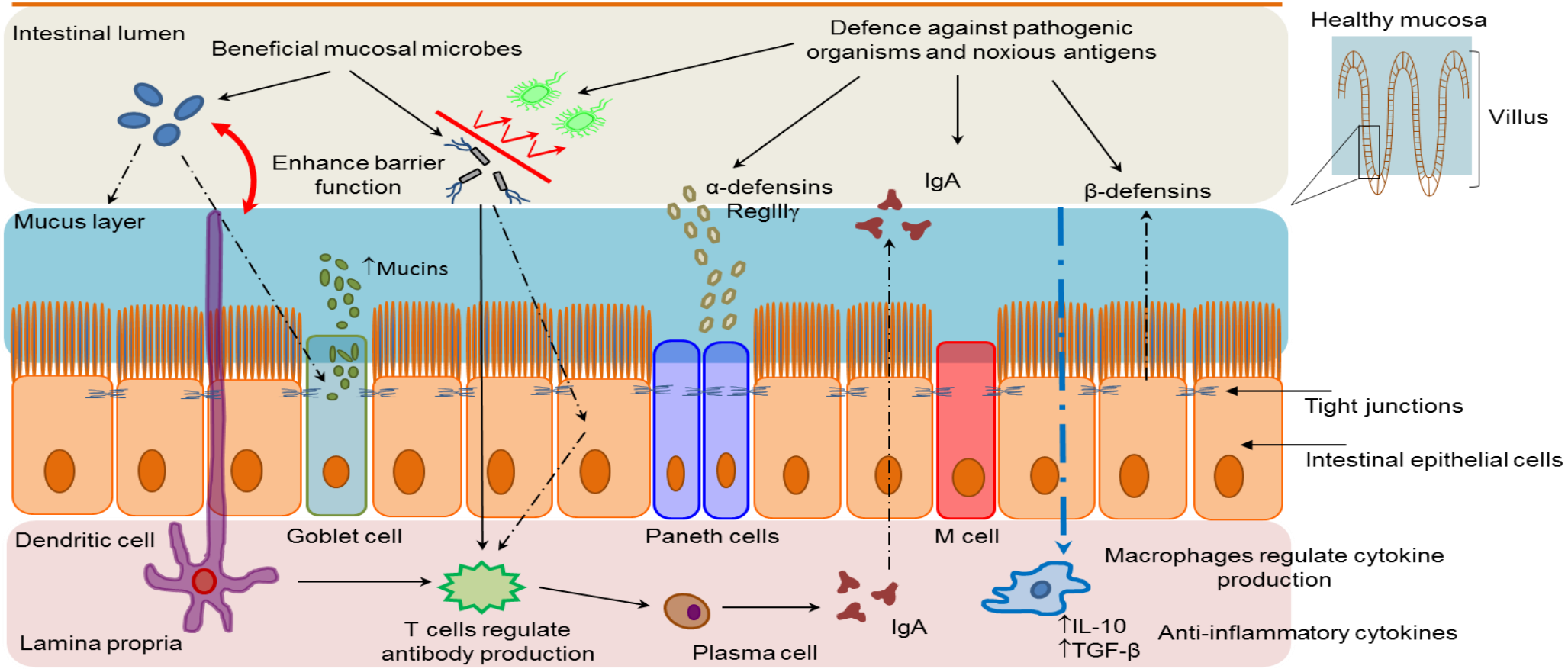

Figure 1.3. Healthy intestinal Mucosa. Diagram shows normal intestinal mucosa. Beneficial microbes support barrier function by regulating immune response and secretion of immunoglobulin (SIgA). Goblet cells secrete mucins, which fortify the mucosal barrier. Paneth cells and intestinal epithelial cells secrete antimicrobial peptides, which decrease pathogens.

1.7.1.1 Barrier function and immunity

The concept of involvement of altered barrier function is not new and was proposed over 70 years ago. Stokes and Pillsbury both dermatologists hypothesized that a breakdown in mucosal barrier function was the causative factor in a number of illnesses including skin conditions and depression (Stokes and Pillsbury 1930). Compromised barrier function has been associated with several diseases that are associated with dysfunctional immune response (Sartor, Rath, Lichtman *et al.* 1996, Bosi, Molteni, Radaelli *et al.* 2006, Camilleri and Gorman 2007, Daley 2014). It has been hypothesised that a stressed and permeable intestinal barrier facilitates the movement of luminal contents, which interact with the mucosal immune system, triggering a cascade of immune responses that manifests as local and systemic inflammatory responses (Hollander 1999, Fasano 2012).

The patency of the mucosal barrier is fundamental in maintaining and mediating contact with the intestinal microbiome (Davidson, Kritas and Butler 2007). Disruption of the defensive role of the mucosal barrier allows access of antigenic compounds to the underlying tissue, where both innate and adaptive immune mechanisms are initiated (Turner 2009) (Figure 1.3). Innate immunity is the first response mechanism and has a significant role in protecting the mucosa from ingested pathogens. Defensins are antimicrobial defence peptides secreted from Paneth cells. They have a broad range of activity against gram positive and gram-negative bacteria, yeasts, fungi and viruses (De Smet and Contreras 2005, Dann and Eckmann 2007). Defensins play a critical role in shaping the microbial ecosystem in the GIT. In humans, defensins HD-5 and HD-6 are secreted in their inactive form and are activated by trypsin (Ghosh, Porter, Shen *et al.* 2002). In mice matrix metalloproteinase (MMP)-7 is needed to activate alpha defensins (Shirafuji, Tanabe,

Satchell *et al.* 2003). Experiments with genetically altered mice lacking MMP-7 expression and mice over expressing HD-5 showed that there was no change in total bacteria. However, there was a change in the ratio of the two mayor phyla Firmicutes:Bacteroidetes (Salzman, Hung, Haribhai *et al.* 2010). In the mice over expressing HD-5, adherence of segmented filamentous bacteria to epithelial cells was reduced, which is significant because segmented filamentous bacteria stimulate secretory (s)IgA and regulate T cell differentiation (Salzman, Hung, Haribhai *et al*. 2010).

Barrier function changes with age (Man, Gicheva and Nicoletti 2014), nutritional status, particular pathologies and both physical and psychological stressors (Davidson, Kritas and Butler 2007). Composition of the intestinal microbiome is also affected by different disease states (Clemente, Ursell, Parfrey *et al*. 2012), diet (Wu, Chen, Hoffmann *et al.* 2011), sex, age and geographical location (Yatsunenko, Rey, Manary *et al*. 2012). A vast amount of stressors can affect both the functioning of the mucosal barrier and the intestinal microbiome.

Interactions between the mucosa and the microbiome are also critical in adaptive immunity. In an experiment with germ free mice, mice were given the microbiota of healthy human donors or the microbiota from healthy mouse donors and then compared to germ free mice. Interestingly, the germ free mice that received the human microbiota had a reduced number of T cells that was comparable to levels in the germ free mice. T helper cells (Th1 and Th17) were measured in the small intestinal lamina propria and were reduced in germ free animals but were increased in the mice that received the healthy mouse microbiota (Chung, Pamp,

Hill *et al.* 2012). Another interesting finding from this study was that although the total bacterial load was similar and relative abundances of bacterial phyla in human and mice microbiota (Firmicutes:Bacteroidetes) were similar, species variations were distinctly different especially in the Firmicutes phyla (Chung, Pamp, Hill *et al*. 2012). Furthermore, when germ free mouse microbiota were infected with *S. enterica* serovar *Typhimurium* they had significantly decreased levels of inflammation and oedema, but maintained a healthy caecal histology 4 days post infection compared with the caecum in germ free mice and germ free mice with healthy human microbiota (Chung, Pamp, Hill *et al.* 2012). These findings imply that host specific bacteria are required for immune system maturation and that host specific endogenous bacteria aid in the health of the mucosal barrier. These examples fit with the hygiene hypothesis that suggests increased microbial contact from a diverse collection of microbes is needed for balanced immune system maturation in early life. This is exemplified by the increased prevalence of disease in adults and children, when the microbiome has been altered by a range of events such as antibiotics, delivery mode (vaginal or caesarean section), and gastrointestinal infection (Spiller and Campbell 2006, Adlerberth and Wold 2009, Russell, Gold, Hartmann *et al.* 2012). These studies also suggest that the colonization of the GIT in humans is sequential, preferential and host specific. More broadly it suggests that a dysregulated immune-mucosa-bacterial interaction is a causative trigger in number of diseases.

1.7.2 Intestinal permeability

Intestinal permeability can be defined as "a functional feature of the intestinal barrier at given sites, measurable by analysing flux rates across the intestinal wall as a whole or across wall components of defined molecules that are largely inert during the process and that can be adequately measured in these settings" (Bischoff, Barbara, Buurman *et al.* 2014). The control of permeability is a fundamental process in preserving homeostasis. There are two distinct routes for the permeation of molecules across the epithelial barrier; the transcellular and the paracellular routes. The transcellular route (Figure 1.4) transports various solutes; nutrients, sugars, amino acids and lipids through the cell. The paracellular route governs the entrance of molecules of different size and charge via the paracellular space in between intestinal epithelial cells (Artis 2008). After enzymatic degradation of carbohydrates, lipids and proteins in the small intestine, transporter complexes located at the apical and basolateral membrane of the colonocyte facilitate the movement of some of these molecules. During health intestinal permeability is stable, meaning there are no signs of inflammation or alterations in function. In disease however, there is a disturbance in the cellular function of permeability that ultimately leads to loss of GIT homeostasis, which can manifest as disease (Bischoff, Barbara, Buurman *et al.* 2014). Maintenance of the mucosal barrier and in particular the control of permeability is a fundamental process preserving GIT homeostasis. Intestinal permeability controls the flow of beneficial molecules across the mucosal barrier while inhibiting those that may be harmful and as such mediates the crosstalk between the luminal contents of the GIT and the internal milieu.

Figure 1.4. Permeation routes across the mucosal membrane.

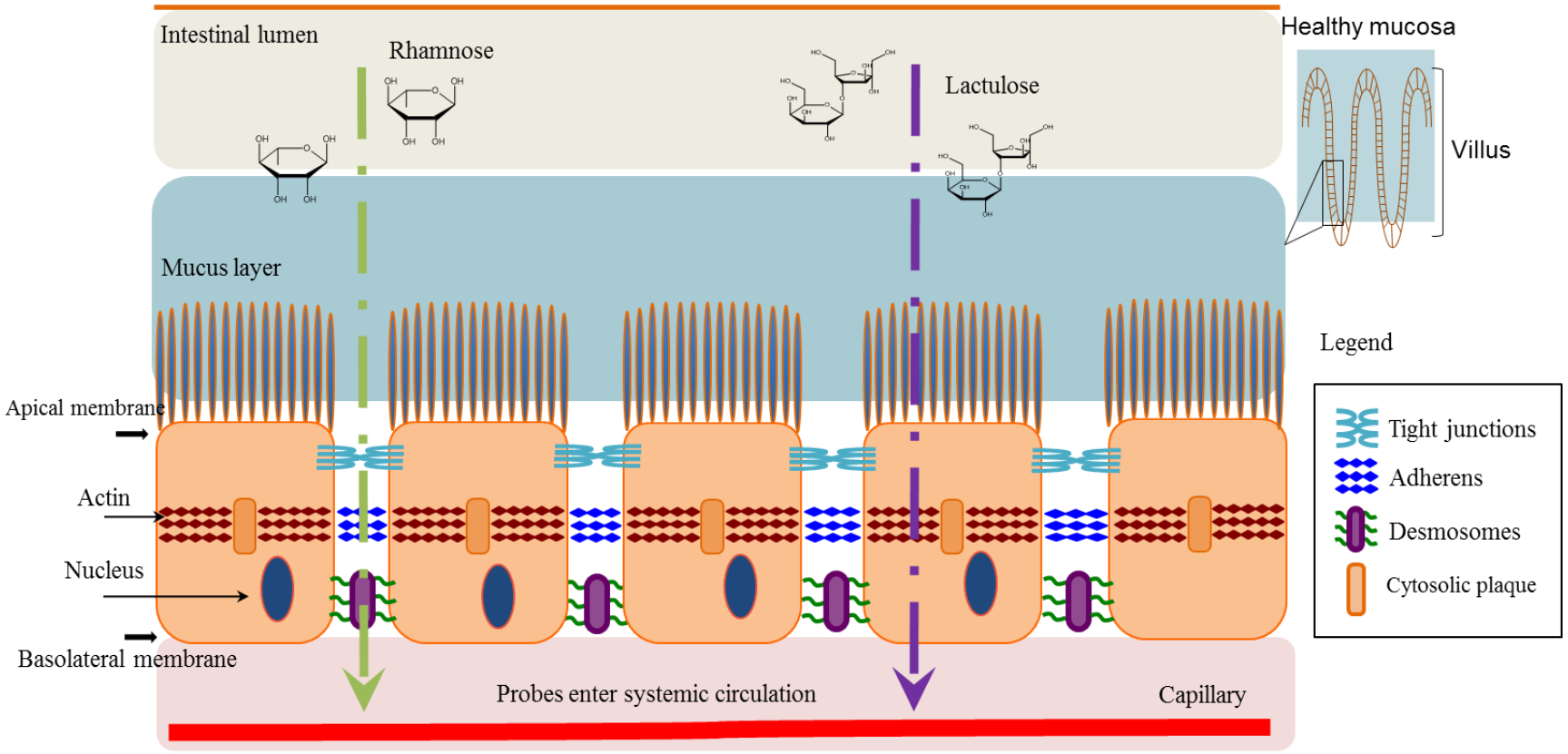

Figure 1.4. Permeation routes across the mucosal membrane. Protein complexes tethering adjacent intestinal epithelial cells lining the gastrointestinal tract. Paracellular route uses spaces between intestinal epithelial cells whereas transcellular route uses specialized transporters to move nutrients across the membrane.

1.7.2.1 Cellular mechanics of the mucosal barrier

At its most basic level, the intestinal mucosa comprises a single layer of intestinal epithelial cells, each tethered by a range of protein complexes that maintain the structural cellular bonds between cells (Giorgio, Miele, Principessa *et al.* 2014) (Figure 1.4). Some of the internal signalling proteins controlling these junction complexes are known (Meng and Takeichi 2009, Ulluwishewa, Anderson, Mcnabb *et al.* 2011). These include myosin light chain kinase, protein kinase C, and the Rho family of small GTPases, which control the interactions between the transmembrane proteins and the actomyosin ring surrounding the apical region of the intestinal epithelial cells.

Tight junctions are paracellular transmembrane proteins, which bind intestinal epithelial cells. They include claudin, occludin, tricellulin and junction adhesion molecule and encircle the apical portion of intestinal epithelial cells providing a tight seal between cells (Günzel and Alan 2013, Suzuki 2013). Claudins are thought to control the movement of ions and molecules according to size and charge (Günzel and Alan 2013). There are currently over 20 claudin proteins identified, with their role split between promoting barrier formation (decreasing intestinal permeability), and involvement in paracellular trafficking (promoting permeability) (Suzuki 2013). Adherens are composed of proteins of the cadherin type, with corresponding intracellular anchoring proteins a-, b-, and c-catenin. These rod shaped proteins anchor the plasma membranes of adjacent cells forming an adhesive bond. These proteins are located below the tight junction complex, and are proposed to work with tight junctions to form a functional unit (Figure 1.4) (Suzuki 2013). Desmosomes are dense connections anchoring epithelial cells via filaments located intracellularly underneath adherens and resist mechanical stress (Figure 1.4) (Garrod and Chidgey 2008). They give

structural and mechanical strength to cellular sheets such as the epithelium lining the GIT. The combination of these structural proteins is responsible for the critical gatekeeper role of the epithelial barrier and facilitates the movement of molecules across intestinal mucosa.

The paracellular domain is a dynamic region that is constantly responding to environmental cues (Ulluwishewa, Anderson, Mcnabb *et al.* 2011). Tight junctions act as "security guards" between the distinct apical and basolateral regions of the intestinal epithelium (Davidson, Kritas and Butler 2007). Together these create the intricate matrix between intestinal epithelial cells, which play a critical role in mucosal permeability, where certain diseases and stressors are likely to affect protein structure leading to a breakdown in the mucosal barrier (Fasano 2012, Günzel and Alan 2013). While our understanding of the molecular structure of tight junctions has increased, gaps still remain in our understanding of the pathobiological factors influencing barrier function.

1.7.3 Mucin secreting goblet cells

Specialised glandular epithelial cells (goblet cells) are distributed throughout the length of the GIT and secrete a range of mucins that protect the mucosa (Artis 2008). Mucins consist of branched or linear oligosaccharides chains connected to a protein backbone (Specian and Oliver 1991). Mucins are excreted as granules from goblet cells and hydrate once in the lumen, forming a viscous layer covering the intestinal mucosa (Figure 1.3). Mucus functions to protect the underlying mucosa from mechanical and chemical stresses (Robbe, Capon, Coddeville *et al.* 2004), while providing adhesion sites for a range of beneficial commensal microorganisms (Stringer, Gibson, Logan *et al.* 2009, Marcobal, Southwick,

Earle *et al.* 2013). Mucins consist of glycoproteins containing glycosylated and non-glycosylated domains (Forstner, Oliver and Sylvester 1995). The main oligosaccharides present in mucins are N-Acetylglucosamine, N-Acetylgalactosamine, fucose, and galactose (Forstner, Oliver and Sylvester 1995). Mucin chains can be terminated by sialic acid and sulphate groups (Forstner, Oliver and Sylvester 1995). Sialic acid derivatives are confined to the small intestine and colon. Sulphated mucins are believed to be less degradable and decrease bacterial translocation (Deplancke and Gaskins 2001, Jass and Walsh 2001).

The mucins secreted throughout the GIT can differ in the level of glycosylation and sulphation, which can modify how commensal microbes ultimately regulate the microecology of the intestinal microbiome (Deplancke and Gaskins 2001). Mucin secretion is stimulated by a range of factors including acid secretion, the presence of microbiota and luminal compounds (Robbe, Capon, Coddeville *et al*. 2004). Secretion of mucus from specific cell lines (human cancer cell line LS180) is stimulated by cytokines such as IL-1, IL-4, IL-6, and TNF-α (Enss, Cornberg, Wagner *et al*. 2000). It is likely that the microbiota produce bioactives that stimulate goblet cell secretion, and affect the bio-dynamics of the mucus layer. In turn, the mucus itself contains a large reservoir of other bioactive substances such as lysozymes, lactoferrin, beta-galectins, trefoil factor peptides and mucosal integrity peptides that influence and modulate the intestinal microbiota (Corfield, Carroll, Myerscough *et al*. 2001). Recently, goblet cells have been shown to secrete bactericidal peptides Ang4, Reg111y and Reg111β (Burger-Van Paassen, Loonen, Witte-Bouma *et al.* 2012), with Reg111y involved in preventing microbial contact with the underlying tissue. Goblet cells may also be able to transfer antigens obtained from the lumen to dendritic cells located in the lamina propria playing a role in modulation of the intestinal microbiome (Mcdole,

Wheeler, Mcdonald *et al.* 2012). The mucus layer therefore plays a dynamic defensive role in protecting the mucosa by providing adhesion sites for commensal bacteria, and a range of bioactive molecules, which act to fortify the intestinal barrier providing mucosal stability.

The microbiome plays a functional role in mucosal immunity by stimulating T cells in the lamina propria leading to the production of sIgA, preventing bacterial colonisation and fostering symbiotic relationships with commensal microorganisms which promote GIT health (Sutherland and Fagarasan 2012). Understanding the patterns of beneficial microorganisms compared with deleterious bacteria during times of mucosal stress may lead to methodologies to ecologically modify the composition of the mucus layer to favour beneficial microorganisms, which promote, protect and enhance barrier function. The endogenous microorganisms of the GIT are of great benefit to the intestinal mucosa and as such any disruption in the composition will likely effect barrier function leading to mucosal stress.

1.8 Selective augmentation of the microbiome

1.8.1 Probiotics

Probiotics are beneficial microbes, most frequently *Lactobacillus* spp. and *Bifidobacterium* spp. and when given in adequate amounts beneficially influence the host (Sanders 2008). Meta-analyses of randomised controlled trials using probiotics provides evidence supporting their use in a range of gastrointestinal disorders suggesting that they can increase barrier function both *in vitro* and *in vivo* (Ewaschuk, Diaz, Meddings *et al.* 2008, Karczewski, Troost, Konings *et al.* 2010, Ohland and Macnaughton 2010, Hering, Richter, Fromm *et al.* 2013). As well as the commonly known probiotics there are several endogenous beneficial

organisms linked with a healthy microbiome, such as, *F. prausnitzii* and *A. muciniphilla* which have been associated with alleviating intestinal inflammation, enhancing barrier function and regulating immune function (Sanders 2008).

In vitro and *in vivo* testing has shown varied ways in which probiotics can beneficially affect barrier function. Studies using Caco-2 and HT29 cell lines have shown several probiotic *Lactobacillus* strains stimulated increased mucin expression. These effects were dependent on the ability of the strain chosen to adhere to the cell monolayer (Mattar, Teitelbaum, Drongowski *et al*. 2002, Macfarlane, Woodmansey and Macfarlane 2005). Other studies have shown that strain selection is a significant factor when determining adherence to intestinal epithelial cells *in vitro*, and therefore the ability to promote barrier function is dependent on this characteristic. Hep-2 and T84 cells lines pre-treated with *L. acidophilus* and *L. rhamnosus* were able to decrease adherence of enterohemorrhagic *E. coli* (EHEC), but other strains of *Lactobacillus* failed to demonstrate this capacity (Sherman, Johnson-Henry, Yeung *et al.* 2005). A study using purified surface proteins from *L. helveticus* were able to show decreased adherence of EHEC and prevent increased intestinal permeability without decreasing *E. coli* growth (Johnson- Henry, Hagen, Gordonpour *et al*. 2007). The use of *Bifidobacterium* spp. isolated from the intestinal tract of infants was found to produce an unidentified lipophilic molecule with the ability to decrease viability of *E. coli* and a range of pathogenic microbes *in vitro* and *in vivo* (Lievin, Peiffer, Hudault *et al*. 2000). Furthermore, the lipophilic molecule was able to destroy intracellular *S. typhimurium* and prevent the invasion of Caco-2 cells by *S. typhimurium* (Lievin, Peiffer, Hudault *et al*. 2000). In studies using *B. longum*, secretion of a non-specified molecule was shown to prevent the adherence of EHEC vero toxin (Kim, Yang, Koo *et al.* 2001). As with *Lactobacillus* spp. strain selection

is critically important in investigating barrier-promoting effects of probiotics and will determine their effectiveness when used in human trials. While the majority of studies regarding probiotics have been carried out in animal models with encouraging results, a great deal still remains unknown of the potential therapeutic mechanisms by which probiotics elicit their influence on the microbiome and the mucosa in humans. These studies indicate the importance of strain selection but also reveal the importance of understanding the endogenous beneficial species present pre-treatment and how to manipulate the composition of the microbiome to influence these microorganisms to promote health.

VSL#3 is a combination probiotic used in the research community in both animal models and human studies. VSL#3 contains 3 strains of *Bifidobacterium*: *B. longum*, *B. infantis*, and *B. breve;* 5 Lactobacilli strains: *L. acidophilus, L. casei, L. delbrueckii*, *L. bulgaricus*, and *L. plantarium;* and *Streptococcus salivarius* subsp. *thermophilus.* In IL-10 gene deficient rats, VSL#3 was able to restore epithelial function, improve barrier function and decrease pro-inflammatory cytokines (Madsen, Cornish, Soper *et al.* 2001). Although, the mechanisms in which probiotic microorganisms exert their influence on the mucosal barrier are still poorly understood, there is increasing evidence that suggests that probiotics have a place in treating a wide variety of illnesses (Vasiljevic and Shah 2008). Currently, there are only a limited number of probiotic microorganisms utilised in studies investigating a range of gastrointestinal illnesses and the effects on barrier function. These include various *Lactobacillus* spp., various *Bifidobacterium* spp., *E. coli* Nissle 1917, *Streptococcus thermophilus* and *Saccharomyces boulardii* (Ohland and Macnaughton 2010). Most, if not all, studies investigating the use of probiotics to influence barrier function use exogenous strains of bacteria isolated from a single human donor at some point. The microbiome is

comprised of between 500 and 1000 microorganisms (Dethlefsen, Mcfall-Ngai and Relman 2007), with an enormous strain variation (Eckburg, Bik, Bernstein *et al*. 2005, Rajilić-Stojanović and Vos 2014). The endogenous bacterial population and the variability in the microbial strains between people make it increasingly important to understand the prevailing microbial composition individually, and how particular patterns of microorganisms and specific strains influence each other, and in turn how this syntrophism may enhance or diminish barrier function.

1.8.2 Probiotics in the treatment of chemotherapy-induced mucositis

Probiotics have shown beneficial effects in various animal studies investigating mucositis (Table 1.3). Indeed, the latest guidelines released for the management of mucositis in the clinical arena by the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) proposed *Lactobacillus* spp. for use in decreasing the severity of diarrhoea (Lalla, Bowen, Barasch *et al.* 2014). In other studies Bowen *et al* 2007, using VSL#3 a combination probiotic, showed reduced weight loss and a decrease in moderate and severe diarrhoea when compared to control rats. There was also diminished apoptosis and increased crypt proliferation in the VSL#3 treated rats (Bowen, Stringer, Gibson *et al*. 2007). Other studies investigating the effects of the probiotics in mucositis have shown *S. thermophilus* prevented the loss of absorptive capacity in the small intestine when measured using the sucrose breath test (SBT), a measure of absorptive capacity in the small intestine (Tooley, Howarth, Lymn *et al*. 2006). The SBT is a validated diagnostic test utilizing ^{13}carbon labelled sucrose (Pelton, Tivey, Howarth *et al.* 2004). The test solution is given orally and the metabolism of which is then measured in breath. Breath is then analysed using isotope ratio mass spectroscopy and

interpreted as an indicator of sucrase activity and thus absorptive capacity of the small intestine (Pelton, Tivey, Howarth *et al*. 2004). These studies are examples of the efficacy of probiotic treatment in chemotherapy-induced mucositis.

Table 1-3: Effects of probiotics in animal models of chemotherapy-induced mucositis.

Experimental group	Probiotic intervention	Parameter measured	Findings	Study
Mice (methotrexate)	*S. thermophilus*	Small intestinal absorption (SBT), Mucosal histology	↑Sucrase activity	(Pelton, Tivey, Howarth *et al.* 2004)
Mice (irinotecan)	VSL#3	Mucosal histology	↓Diarrhoea ↓Weight loss ↓Crypt proliferation	(Bowen, Stringer, Gibson *et al.* 2007)
Rat (methotrexate)	*L. bulgaricus* *S. thermophilus* *L. johnsonii*	Small intestinal permeability, Mucosal histology	↓Permeability ↓Mucosal damage	(Southcott, Tooley, Howarth *et al*. 2008)
Rat (5-fluorouracil)	*E. coli* Nissle (supernatant) *L. fermentum* (supernatant)	Small intestinal absorption (SBT), Mucosal histology	↓Mucosal damage	(Prisciandaro, Geier, Butler *et al*. 2011)
Mice (5-fluorouracil)	*S. boulardii*	Intestinal permeability, Cytokines	↓TNF-a and IL-6 ↓Permeability	(Justino, Melo, Nogueira *et al*. 2014)

There is marked changes in both the mucosal architecture and the intestinal microbiome during chemotherapy-induced mucositis (Stringer, Gibson, Logan *et al*. 2008). As such, changes in the microbiota during chemotherapy may be a target of focused bacterial therapy in the form of probiotics. Studies in human mucositis sufferers have shown beneficial effects of certain species of probiotics (Gibson, Keefe, Lalla *et al.* 2013) (Table 1.4). A large study in patients receiving 5-fluorouracil as the chemotherapeutic agent were assigned *L. rhamnosus* GG with or without chemotherapy (Österlund, Ruotsalainen, Korpela *et al.* 2007). The treatment group reported decreased grade 3 and 4 diarrhoea, and reductions in abdominal discomfort (Österlund, Ruotsalainen, Korpela *et al*. 2007). Treatment with *L. rhamnosus* GG did not alter levels of the chemotherapeutic. The findings of this study recommend the usage of *L. rhamnosus* GG as an adjunctive therapy in the management of diarrhoeal symptoms. Another large study in human radiotherapy patients found that VSL3# reduced diarrhoeal severity compared to the placebo group (Delia, Sansotta, Donato *et al.* 2007). Others have reported in radiotherapy patients receiving *L. casei* that although diarrhoeal severity was not altered compared to placebo, a significant effect on stool consistency was reported (Giralt, Regadera, Verges *et al.* 2008). These studies and recommendations from the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO)- working group indicate that probiotics have a place in the treatment regimens of patients undergoing chemotherapy, and probiotics may help ameliorate the symptoms of mucositis.

Table 1-4: Human trials with probiotics in the treatment of chemotherapy-induced mucositis.

Experimental group	Study type	Probiotic intervention	Parameter measured	Findings	Study
Mucositis patients (n=98) Controls (n=52)	Open-label, prospective, randomised, phase III	*L. rhamnosus* GG	Frequency of severe diarrhoea	↓Grade 3 and 4 diarrhoea ↓Bloating	Finland, (Österlund, Ruotsalainen, Korpela *et al*. 2007)
Radiotherapy (n=243) Placebo (n=239)	Double-blind, placebo-controlled trial	VSL#3	Incidence and severity diarrhoea,	↓Grade 3 and 4 diarrhoea	Italy, (Delia, Sansotta, Donato *et al*. 2007)
Radiotherapy patients (n=44) Placebo (n=41)	Placebo-controlled, double-blind, randomized clinical trial	*L. casei*	Diarrhoea severity	No change ↑Stool consistency	Spain, (Giralt, Regadera, Verges *et al.* 2008)
Radiotherapy (n=11) Controls (10)	Randomized control trial	*L. acidophilus*	Incidence of diarrhoea	↓Diarrhoea	Finland, (Salminen, Elomaa, Minkkinen *et al*. 1988)
Radiotherapy (n=32) Placebo (n=31)	Randomized, double-blind, placebo-controlled study	*L. acidophilus* *B. bifidum*	Incidence and severity diarrhoea	↓Diarrhoea ↑Stool consistency	Thailand, (Chitapanarux, Chitapanarux, Traisathit *et al*. 2010)

1.8.3 Probiotics in the treatment of virally induced diarrhoea.

AGE is a life threatening illness in young children where the loss of fluids must be halted to prevent severe dehydration and possible death. The use of probiotics to achieve this aim has been studied in many countries where diarrhoeal illness is still a major killer of children under 5 years of age (Table 1.5). The most widely studied probiotic for the treatment of acute diarrhoea in the paediatric arena is *Lactobacillus* GG (LGG). Many studies have shown the efficacy of LGG to decrease duration and severity of diarrhoea this appears to be when the causative agent is RV (Basu, Paul, Ganguly *et al*. 2009, Sindhu, Sowmyanarayanan, Paul *et al*. 2014), whereas some studies showing no difference at all (Rosenfeldt, Michaelsen, Jakobsen *et al*. 2002, Salazar-Lindo, Miranda-Langschwager, Campos-Sanchez *et al*. 2004, Ritchie, Brewster, Tran *et al.* 2010). Other studies have shown that the type of pathogen influences clinical parameters such as reduced intestinal permeability as measured with the dual sugar permeability test in patients with cryptosporidium infection when treated with LGG (Sindhu, Sowmyanarayanan, Paul *et al.* 2014) The above studies are examples of probiotic interventions into acute gastroenteritis in children. The above studies indicate that probiotic treatment into acute gastroenteritis is effective in reducing severity and duration of diarrhoea, however more studies are need to elucidate the mechanisms of action of probiotics in acute gastroenteritis.

Table 1-5: Effects of probiotics in the treatment of acute gastroenteritis in children.

Experimental group (children)	Study type	Probiotic intervention	Parameter measured	Findings	Study
Treatment group (n=30) Placebo (n=39)	Randomised double-blind placebo controlled	*L. rhamnosus* 19070-2 *L. reuteri* DSM 12246	Diarrhoea severity Hospital stay	No change in diarrhoeal severity ↓Hospital stay	Denmark, (Rosenfeldt, Michaelsen, Jakobsen *et al*. 2002)
Mixed pathogens (n=71) Placebo (n=78)	Randomized double-blind placebo controlled	*L. casei* GG	Diarrhoea severity	No change between groups	Peru, (Salazar-Lindo, Miranda-Langschwager, Campos-Sanchez *et al*. 2004)
Rotavirus (n=113) Placebo (n=111)	Randomized double-blind placebo-controlled	VSL#3	Diarrhoea severity	↓Diarrhoea severity ↓Oral rehydration required	India, (Dubey, Rajeshwari, Chakravarty *et al.* 2008)
Acute watery diarrhoea (n=364) Controls (n=185)	A Randomized Controlled Trial	*L. rhamnosus* GG	Diarrhoea severity	↓Diarrhoea severity ↓Diarrhoea duration	India, (Basu, Paul, Ganguly *et al.* 2009)
Rotavirus (n=33) Placebo (n=31)	Randomised double-blind placebo-controlled	*L. casei* GG	Diarrhoea severity SBT	No changes in diarrhoea No changes in SBT results	Australia, (Ritchie, Brewster, Tran *et al.* 2010)

Table 1.5: continued

Experimental group (children)	Study type	Probiotic intervention	Parameter measured	Findings	Study
Rotavirus (n=44) Placebo (n=20)	Randomized double-blind controlled	*S. boulardii* ; and combination probiotics (*L. acidophilus*, *L. rhamnosus*, *B. longum* and *S. boulardii*)	Diarrhoea severity	↓Diarrhoea severity	Boliva, (Grandy, Medina, Soria *et al*. 2010)
Acute diarrhoea (n=90) Placebo (n=86)	Randomized, double-blind, placebo-controlled	*S. boulardii*	Diarrhoea severity	↓Diarrhoea severity	Brazil, (Corrêa, Penna, Lima *et al*. 2011)
Watery diarrhoea (n=87) Controls (n=88)	Open labelled, randomized controlled trial	*L. rhamnosus* GG	Duration of diarrhoea	↓Duration of diarrhoea	India, (Aggarwal, Upadhyay, Shah *et al.* 2014)
Rotavirus (n=45) Cryptosporidial diarrhoea (n=20) Placebo (n=59)	Randomized, double-blind, placebo-controlled	*L. rhamnosus* GG	Intestinal permeability Diarrhoea severity (Vesikari score) IgG levels	↓Intestinal permeability in Cryptosporidial group only ↓Diarrhoea severity in rotavirus group only ↓IgG in rotavirus group only	India, (Sindhu, Sowmyanarayanan, Paul *et al.* 2014)

Table 1.5 continued

Experimental group (children)	Study type	Probiotic intervention	Parameter measured	Findings	Study
Rotavirus (n=42) Salmonella (n=48) Unknown (n=69)	Open label randomized controlled trial	BIO-THREE© (*Enterococcus faecalis* T-110, *Clostridium butyricum* TO-A *Bacillus mesentericus* TO-A	Diarrhoea severity (Vesikari score)	↓Diarrhoea severity (rotavirus group)	China, (Huang, Liu, Chen *et al.* 2014)

1.8.4 Probiotics in the treatment of irritable bowel syndrome

An effective treatment for IBS still remains elusive, with symptoms managed as they arise. This drives many patients to seek alternative therapies to control symptoms (Didari, Mozaffari, Nikfar *et al*. 2015). Some of these therapies involve the consumption of probiotics. Animal models investigating probiotic consumption have shown efficacy in a number of models of damage however, there are few animal models of general IBS. Wang *et al* 2014, using a parasite (*Trichinella spiralis*) to infect mice and induce a transient change in the intestinal environment (similar to post infectious IBS) have shown a combination of probiotics effective at increasing barrier function and other intestinal parameters (Wang, Gong, Wang *et al.* 2014). The mice were given a combination of probiotics or a single probiotic. *B. longum*, *L. acidophilus* and *S. faecalis*, *B. longum* and, *L. acidophilus* but not *S. faecalis* decreased contractile responses to acetyl choline (ACH) in longitudinal muscle fibres (Wang, Gong, Wang *et al.* 2014). Measurements of intestinal permeability (D-lactate in blood) were decreased by *B. longum* and *L. acidophilus*, and the combination mixture but not by *S. faecalis* alone (Wang, Gong, Wang *et al.* 2014).

The efficacy of the probiotics and the usefulness in treating IBS is gaining momentum. There is growing evidence that they may be useful in the management of IBS symptoms (Ford, Quigley, Lacy *et al.* 2014, Didari, Mozaffari, Nikfar *et al.* 2015). Studies with probiotics in the treatment of IBS have shown a degree of variability (Table 1.6). One study showed that particular phenotypes of IBS responded better than others for example, IBS patients with their symptoms attributable to post infectious gastroenteritis or antibiotic usage showed better response rates than standard IBS with no known pre-infection or medication (Kruis, Chrubasik, Boehm *et al.* 2012). Other studies have shown specific probiotics are effective

in the treatment of certain phenotypes of IBS. In IBS-C patients, *B. lactis* decreased abdominal distension and increased orocaecal and colonic transit time (Agrawal, Houghton, Morris *et al.* 2009). These studies indicate that probiotics are an emerging therapeutic option in the treatment of IBS symptoms. Further research is needed to understand the complex role of the probiotics used as therapeutic agents, and the effect on the endogenous microbiome.

Currently, there are there are no prospective studies that measure changes in the microbiome in specific disease states where mucosal stress is evident such as chemotherapy induced mucositis, viral diarrhoea and IBS. Nor are there interventions using randomised double blind trials to actively modify those changes by administering a probiotic and measure favourable changes in parameters of a mucosa under stress. Given the equivocal efficacy data for a variety of interventions that are believed to modify both the endogenous microbiome composition and in some cases parameters related to mucosal stress it would seem worthwhile to examine the differences in the individual microbiomes at different stages of disease and to determine whether this and the host response parameters constitute subsets of gut dysfunction with a differential responder and non-responder profile. The first step in this process is to measure profiles and activities in different gastrointestinal diseases in order to design intervention trials with a personalized approach. Despite these deficiencies convincing data is beginning to emerge emphasizing the critical role of the intestinal barrier and in particular intestinal permeability for maintenance and promotion of health and the prevention of disease.

Table 1-6: Probiotic interventions in IBS.

Experimental group	Study type	Probiotic intervention	Parameter measured	Findings	Study
IBS (n=25) Controls (n=27)	Randomized, double-blind placebo-controlled, trial	*L. plantarum* DSM 9843	Visual analog scales	↓Pain ↓Flatulence	Sweden, (Nobaek, Johansson, Molin *et al*. 2000)
IBS-C (n=17) Controls IBS-C (n=15)	Randomized, double-blind, controlled, parallel-group	*B. lactis* DN-173 010	Abdominal distension Transit time	↓Distension ↑transit time	UK, (Agrawal, Houghton, Morris *et al.* 2009)
IBS-C (n=194) Controls IBS-C (n=184)	Randomized, control trials	*L. casei* Shirota, *E. coli* Nissle, *L. casei* Lcr35 *L. rhamnosus* GG,	Defecation frequency Stool consistency	↑Frequency ↑Consistency	Multiple countries, (Chmielewska and Szajewska 2010)
IBS (n=33) Controls (n=34)	Randomized controlled study	Cultura; active © (*L. bulgaricus* *S. thermophilus* *L. paracasei*, spp. *paracasei* F19, *L. acidophilus* La5 *B. lactis* Bb12)	Improvement in IBS symptoms	No change	Sweden, (Simrén, Öhman, Olsson *et a*l. 2010)

Table 1.6 continued

Experimental group	Study type	Probiotic intervention	Parameter measured	Findings	Study
IBS (n=105) Placebo (n=95)	Double blind, placebo-controlled study	*L. plantarum* 299v	Abdominal bloating Abdominal pain	↓Bloating ↓Pain	India, (Ducrotté, Sawant and Jayanthi 2012)
IBS (n=54) Controls (n=49)	Randomized double-blind study	MUTAFLOR® *E. coli* Nissle 1917	Satisfaction scale Quality of life variables	No change	Germany, (Kruis, Chrubasik, Boehm *et al*. 2012)
IBS (n=45) Placebo (n=29)	Randomized, double-blind, placebo-controlled intervention clinical trial	*L. plantarum* CECT7484 *L. plantarum* CECT7485) *Pediococcus acidilactici* CECT7483	IBS-quality of life	↑Quality of life	Spain, (Lorenzo-Zúñiga, Llop, Suárez *et al.* 2014)
IBS (n=25) Placebo (n=24)	Randomized, double-blind, placebo-controlled trial	*B. longum,* *B. bifidum,* *B. lactis,* *L. acidophilus,* *L. rhamnosus* *S. thermophilus*	IBS symptoms Pain	↓IBS symptoms	Korea, (Yoon, Sohn, Lee *et al.* 2014)

Table 1.6 continued

Experimental group	Study type	Probiotic intervention	Parameter measured	Findings	Study
IBS (n=100) Placebo (n=52)	Randomised, double-blind, placebo-controlled trial	Symprove© (*L. rhamnosus* NCIMB 30174, *L. plantarum* NCIMB 30173, *L. acidophilus* NCIMB 30175, *E. faecium* NCIMB 30176)	IBS-symptom severity score	↓Pain ↑Bowel habit satisfaction	UK, (Sisson, Ayis, Sherwood *et al*. 2014)
IBS (n=21) Placebo (n=19)	Randomized, double-blind placebo-controlled, trial	*B. lactis* W52, *L. casei* W56, *L. salivarius* W57, *Lactococcus lactis* W58, *L. acidophilus* NCFM, *L. rhamnosus* W71	Visceral hypersensitivity	No change	Netherlands, (Ludidi, Jonkers, Koning *et al*. 2014)

1.9 Conclusions

The role played by a pre-existing gut microbiome in the susceptibility to the development and severity of a range of gastrointestinal disorders is under studied. Furthermore, the influence that specific bacterial populations exert on a range of healthy gut physiology, and on a propensity to dysfunction is in its infancy. A first step in this endeavour is the documentation of compositional changes indicative of an altered and imbalanced microbiome, prior to insult or infection, during disease and in the recovery phases. The link between differing degrees of mucosal stress and indicators to detect and monitor these changes now needs to be integrated with the burgeoning technical capabilities for measuring the changes in microbiome composition, and in the future, the activity of these microbial ecosystems.

Compromised barrier function and increased intestinal permeability are indicators of a stressed mucosa and are critically significant factors affecting GIT homeostasis. They are becoming increasingly important to understand in the context of disease initiation and progression, both in the GIT and extra-intestinally. Furthermore, the failure of the intestinal barrier to maintain its critical gatekeeper role endangers the host by exposing the mucosal immune system to a vast array of bacterial metabolites and microorganisms, which may in turn generate a complex immune response leading to pathology. Understanding a stressed mucosa and factors effecting barrier function and permeability have the potential to help reduce symptoms of this increasingly expensive area of medical treatment. Understanding the link between a stressed mucosa and the microbiome may yield more clinically relevant diagnostics, while providing information regarding microbial patterns indicative of a specific disease states. This information may lead to targeted

therapeutic interventions aimed at altering the composition of the microbiome to a balanced state (which is yet to be clearly defined) with the overall goal of restoring mucosal integrity during times of mucosal stress for the promotion of health.

1.10 Aims of thesis

Major aims

The aims of this research are to relate conditions where a stressed mucosa is evident and find relationships with non-invasive markers of mucosal dysfunction, fermentative activity (intestinal gases) and patterns of microbes that may be able to help define a stressed mucosa in a clinical setting. This thesis aims to determine the link between mucosal stress and the intestinal microbiome and defined parameters of altered barrier function by investigating three separate models representing different degrees of gastrointestinal mucosal stress. This will involve a retrospective analysis of an *in vivo* animal model and clinical patient samples.

Aim 1:

Characterise the microbiota in a tumour-bearing Dark Agouti rat model of chemotherapy-induced mucositis and relate microbiology to caecal and colonic histology and clinical signs of stressed mucosa (diarrhoea).

Aim 2.

Determine the relationship of the microbiota in two different viral infections compared to healthy controls and relate microbial changes in the viral groups to clinical symptoms of mucosal stress (diarrhoea).

Aim 3:

Determine the relationship of intestinal permeability, intestinal inflammation (markers of stressed mucosa) and specific bacteria of the intestinal microbiome in a cohort of well-characterized IBS patients with respect to clinical symptoms (diarrhoea and constipation).

Aim 4:

Determine the relationship between intestinal permeability and intestinal inflammation, microorganisms controlling the hydrogen economy and intestinal gases measured non-invasively in breath, (CH_4 and H_2) in a cohort of IBS patients with mixed symptomatology (diarrhoea, constipation and mixed).

Chapter 2.0 Alterations in the caecal and colonic microbiota contribute to mucosal damage in chemotherapy-induced mucositis

2.1 Preface

Damage to the mucosa has been reported to be more severe in tumour-bearing animals, therefore this was deemed a better model for investigating potential targets to alleviate chemotherapy side effects in humans. The most important observations from this chapter are the regional differences, and that the regional compositional changes in the microbiome could be assessed in the light of localized mucosal damage and subsequent dysfunction. The potential pathogen to beneficial bacteria ratio (exemplified in one iteration by *E. coli:Bifidobacterium* spp.) could be correlated with effects on mucosal integrity at different stages of damage and repair cycles. In these studies the time of most damage coincided with elevation of *E. coli* and SRB levels and diminished beneficial species. As recovery began these values started to normalize. This study has shown that there are marked regional differences in the microbiome of rats undergoing chemotherapy compared to controls, and that these compositional shifts in the microbiome may exacerbate chemotherapy induced mucositis. These results also suggest that altering the microbiome to promote a more balanced beneficial to pathogenic bacterial composition prior to chemotherapy may be a therapeutic option.

2.2 Abstract

Introduction

Chemotherapy-induced mucositis is a debilitating, and sometimes life threatening consequence of cancer treatment causing mucosal injury. Changes to the intestinal microbiome and mucosa are characterised by clinical symptoms including diarrhoea, bloating, ulceration, vomiting and nausea.

Material and Methods

Tumour bearing dark agouti rats were treated with irinotecan and monitored for 120 h. Rats were euthanized at specific time points to analyse potential changes in the microbiota from the caecum and colon and the mucosal architecture following administration of the chemotherapeutic agent.

Results

Irinotecan-treated rats showed greatest incidence of diarrhoea at 72 h (68%), and greatest severity at 96 h (16.7% Grade 3). Histological damage also occurred with greatest severity 24-96 h. Caecal *Bifidobacterium* spp. significantly decreased at 96 h (mucosal damage and diarrhoea present) compared to control (p=0.01), and at 48, 72 and 96 h compared with 120 h (histological repair) (p=0.02, 0.05 and 0.02, respectively). *Lactobacillus* spp. decreased significantly at 96 h compared to 6 h (p=0.05). *E. coli* significantly increased at 72 h (mucosal damage, diarrhoea) compared with controls (p=0.008) and 6 h (p=0.03). Sulphate-reducing bacteria (SRB) significantly increased at 96 h compared with controls (p=0.03). Colonic *Bifidobacterium* spp. significantly decreased at 48 and 72 h compared with 6 h (p=0.016 and p=0.017, respectively). *Lactobacillus* spp. decreased significantly at 72 h compared with controls (p=0.02), and

120 h (p=0.005). *E. coli* significantly increased at 72 h compared with controls (p=0.04). *Bacteroides* spp. was unchanged in the caecum or colon.

Conclusions

Decreased *Lactobacillus* and *Bifidobacterium* spp., and increased *E. coli* and SRB may modulate severity of damage and recovery time following irinotecan. Similar data may be used as a biomarker for severity of damage. Understanding changes in microorganisms following chemotherapy is likely to play a role in defining a patient-specific approach to the treatment of mucositis.

2.3 Introduction

Chemotherapy-induced mucositis is a debilitating consequence of cancer therapy resulting in major mucosal injury (Stringer, Gibson, Bowen *et al.* 2007). Mucositis presents with symptoms present from the oral cavity (pain and erythema, dysphagia and food aversion) to the intestine (abdominal pain, diarrhoea, ulceration and bleeding). (Keefe, Schubert, Elting *et al.* 2007, Wardill, Bowen, Al-Dasooqi *et al.* 2014). Furthermore, mucositis severely compromises patient prognosis, limiting treatment protocols and decreasing quality of life and survival rates (Stringer 2013). The most clinically relevant symptoms such as diarrhoea can be treated acutely with anti-diarrhoeal medications. However, these are not always successful. The Mucositis Study Group of the MASCC/ISOO recently suggested that probiotic treatment containing *Lactobacillus* spp. may be beneficial for prevention of chemotherapy and radiotherapy-induced diarrhoea (Lalla, Bowen, Barasch *et al.* 2014). However, little is understood about the compositional shifts occurring in the intestinal microbiome in different regions of the GIT during chemotherapy-induced mucositis.

The intestinal microbiome is now widely regarded as an essential organ, performing critical functions in the maintenance of health (Xu and Gordon 2003). Microbial changes caused by chemotherapy alter the composition of the microbiome, dysregulating bacterial homeostasis, and increasing the toxicity of some chemotherapeutic agents such as irinotecan (Stringer, Gibson, Logan *et al.* 2008). The microbiome is increasingly recognised as having a major effect on the trajectory of mucositis by affecting intestinal permeability, modulating the inflammatory process and affecting the mucus layer (Van Vliet, Harmsen, De Bont *et al.* 2010). In a rat model of irinotecan-induced mucositis, the shift in composition of the microbiome during cancer treatment favours potentially

pathogenic bacteria such as *E. coli*. at the expense of the mucosally protective bacteria (*Bifidobacteria* and *Lactobacilli*) (Stringer, Gibson, Logan *et al.* 2008). In humans, decreases in the anti-inflammatory commensal bacteria *F. prausnitzii,* and the mucosally beneficial *Bifidobacterium* and *Lactobacillus* spp. occur alongside increases in potential pathogens, such as *E. coli* and *Clostridium difficile,* during chemotherapy compared to healthy controls (Zwielehner, Lassl, Hippe *et al.* 2011). Compositional shifts may impact the functioning of the microbiome, and alter immune responses to the new microbial composition elicited by cancer treatments generating increased mucosal dysfunction.

Irinotecan is a chemotherapeutic agent commonly used in conjunction with other anti-cancer drugs to enhance efficacy (Ding, Wu, Jiang *et al.* 2014). Irinotecan is used in the treatment of solid tumours, primarily disrupting DNA topoisomerase 1 (Andoh, Ishii, Suzuki *et al.* 1987). Irinotecan is a pro-drug and, through hepatic and gastrointestinal carboxylesterases is converted to the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38). SN-38 is then conjugated to the less toxic SN-38 glucuronide (SN-38G) and excreted into the duodenum via bile. Intestinal bacteria exhibiting β-glucuronidase activity such as *E. coli*, can hydrolyse SN-38G back to the toxic metabolite SN-38, consequently increasing its toxicity in the intestine (Takasuna, Hagiwara, Hirohashi *et al.* 1996).

The mucosa of the gastrointestinal tract is the largest cellular surface in continuous direct contact with a vast number of microbes, the internal milieu and the external luminal environment (Hooper and Macpherson 2010). The intestinal microbiota is essential for host homeostasis and is positively correlated with human health (Rajilić- Stojanović and

Vos 2014). Irinotecan has been shown to affect the composition of the intestinal microbiome, increasing bacteria with β-glucuronidase activity and possibly increasing mucosal stress (Stringer, Gibson, Logan *et al*. 2009, Zwielehner, Lassl, Hippe *et al*. 2011, Montassier, Batard, Massart *et al.* 2014). A stressed mucosa may lead to increased intestinal permeability, and disrupted barrier function exposing immune cells embedded in the underlying tissue to a vast array of metabolites and microorganisms in the GIT (Davidson, Kritas and Butler 2007). This may trigger an inflammatory cascade in the mucosa, already under stress from cytotoxic agents. Non-invasive indicators of mucosal stress may help in developing new treatment protocols targeted at mucosal repair. Understanding microbial changes during chemotherapy may help identify which microbiota may potentially fortify a stressed mucosa resulting in improved barrier function and accelerated recovery times.

The primary aim of this study was to examine the relationship between temporal damage response parameters of the intestinal mucosa and changes to the luminal microbiota in the caecum and the colon of tumour-bearing Dark Agouti rats treated with irinotecan It is important to note that all rats in this study were tumour bearing, creating a baseline immunological response, which may have inherently altered the microbiome. This particular model of chemotherapy-induced mucositis was chosen for clinical relevance in the oncology setting. This study is anticipated to generate data with respect to the interface between the intestinal microbiota and the extent of damage following irinotecan.

2.4 Materials and Methods

2.4.1 Animals and Ethics

Archived samples were utilised for this study The Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS) and The University of Adelaide approved this study (M-2010-118A). The study complied with the National Health and medical Research Council (NHMRC), Australian code for the care and use of animals for scientific purposes 8th edition (2013). All experiments were carried out in inbred tumour-bearing female Dark Agouti (DA) rats, weighing 150-170 g. Rats were housed in Perspex cages in the IMVS animal facility regulated at 22 ± 1°C and subject to a 14:10 h light-dark cycle. Animals were monitored four times daily for the duration of the study.

2.4.2 Experimental design

All rats received a subcutaneous injection of tumour inoculum (Dark Agouti mammary adenocarcinoma, DAMA) of 150 µL of cells at a concentration of 2.0 × 107 cells/mL in 0.5 mL sterile PBS, into each flank as previously described (Wardill, Bowen, Al-Dasooqi et al. 2014). Tumours were allowed to grow for 7 days prior to rats receiving irinotecan. Rats were randomly assigned to groups representing time following irinotecan administration. Final numbers used for analyses were: control (n=6), 6 h (n=5), 24 h (n=5), 48 h (n=5), 72 h (n=8), 96 h (n=5), and 120 h (n=6). Irinotecan (supplied by Pfizer, Kalamazoo, Michigan, USA) was activated with the vehicle sorbitol lactic acid buffer (45 mg/mL sorbitol, 0.9 mg/mL lactic acid, pH 3.4). Each experimental rat received 0.01 mg/kg of atropine subcutaneously, followed by 175 mg/kg irinotecan or vehicle intraperitoneally. Rats were humanely killed under deep anaesthesia with 3% isofluorane in 100% O2, using exsanguination and cervical dislocation.

2.4.3 Diarrhoea assessment

Diarrhoea severity was scored using a previously described grading system (Gibson, Bowen, Inglis et al. 2003), where 0 = no diarrhoea, 1 = mild diarrhoea (staining of anus), 2 = moderate diarrhoea (staining over top of the legs and lower abdomen) and 3 = severe diarrhoea (staining over legs and higher abdomen, and associated with continual oozing).

2.4.4 Tissue preparation and histopathology

The GIT was removed from each rat (from pyloric sphincter to rectum), separated into small intestine and colon and flushed with sterile chilled isotonic saline. Samples of jejunum and colon (approximately 1cm in length) were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax. Samples were stained with routine haematoxylin and eosin staining as described previously (Stringer, Gibson, Logan *et al*. 2009). Briefly, sections were dewaxed in xylene and rehydrated in 100% and 90% ethanol. Sections were stained in Lillie Mayer's haematoxylin for 10 min, washed in tap water and differentiated in acid-alcohol. Sections were then blued in ammonia water and counterstained with eosin. Sections were dehydrated, cleared and mounted. Slides were analysed with NanoZoomer digital pathology software (Histalim, Montpellier). Individual parameters of villous blunting, villous fusion (SI) and crypt ablation (SI and colon) were analysed by grading from 0 to 3, with 0 = no change from standard histological structure, 1 = up to one third of tissue affected, 2 = between one and two thirds of tissue affected, and 3 = greater than two thirds of tissue affected based on these markers of histological damage (Logan, Gibson, Bowen et al. 2008).

2.4.5 DNA extraction from colon and caecal samples

Bacterial DNA was extracted from 0.25 g of caecum and colon contents using the bead beating plus column method described by Yu and Morrison (Yu and Morrison 2004). Briefly, caecal and colonic contents were thawed on ice, then homogenised with garnet beads (0.70 mm) in lysis buffer (29.22 g/L NaCl, 6.075 g/L Tris base, 18.612 g/L ethylenediaminetetraacetic acid [EDTA], and 40 g/L sodium dodecyl sulfate [SDS], all purchased from Sigma Aldrich, in milliQ water, pH 8). Samples were then incubated at 70°C for 15 min. Precipitation of nucleic acid was achieved with a set of incubation and centrifuging steps using ammonium acetate, isopropanol and 70% ethanol. Nucleic acid pellet was dissolved in Tris-EDTA (TE) buffer (12.1 g/L Tris base and 3.72 g/L EDTA in milliQ water, pH 8). Total DNA concentration and purity (260/280 nm) were determined using a Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, Scoresby, Australia). DNA was diluted to a working concentration of 5 ng/µL.

2.4.6 Quantitative real time PCR

Microbiota was quantitated using quantitative real time PCR (qPCR) with previously optimised primer sets, targeting V3 region of bacterial 16s rRNA genes (Table 2.1). Optimised annealing times and temperatures were used for each set of primers (Table 2.1) for 45 cycles, except for *Bacteroides* spp. where 35 cycles were used. All qPCR analyses were performed on a Rotorgene Q (QIAGEN, Australia), using Quantitect® SYBR green mastermix (Qiagen) as previously described (Stringer, Gibson, Logan et al. 2008). PCR mixture for each reaction consisted of 5µL of 2x Quantitect® SYBR green mastermix (Qiagen), 2.5 ng/µL of each primer (forward and reverse, 3 µL total) and 10 ng (2 µL total) of sample in a total volume of 10 µL. Each sample was tested in triplicate.

Standard curves were constructed using 5 serial dilutions of known bacterial DNA. Data was analysed using Rotor Gene Q-software (QIAGEN, Australia).

2.4.7 Statistical analysis

Colonic and caecal levels of selected bacteria from the control group were compared with colonic and caecal levels of selected bacteria from rats at 6 h, 12 h, 24 h, 48 h, 72 h, 96 h and 120 h post irinotecan administration, and between rats in the treatment groups at the various time intervals. Data were analysed using Mann Whitney test U with Bonferroni correction applied to correct for Type 1 error. Data was analysed using Graphpad Prism version 6 (GraphPad® Software, California, U.S.A). $p<0.05$ was considered significant, with all data shown as mean ± SEM.

2.5 Results

2.5.1 Irinotecan induces diarrhoea

Greater than 50% of rats administered irinotecan developed diarrhoea of varying degrees. Control rats did not develop diarrhoea at any time point. Diarrhoea first appeared at 6 h with the greatest incidence occurring at 72 h post irinotecan administration, where 68% of rats had diarrhoea (Grade 1-3), and greatest severity occurring at 96 h post irinotecan administration with 16.7% of rats having Grade 3 diarrhoea (Figure 2.1). Diarrhoea was resolved by 120 h.

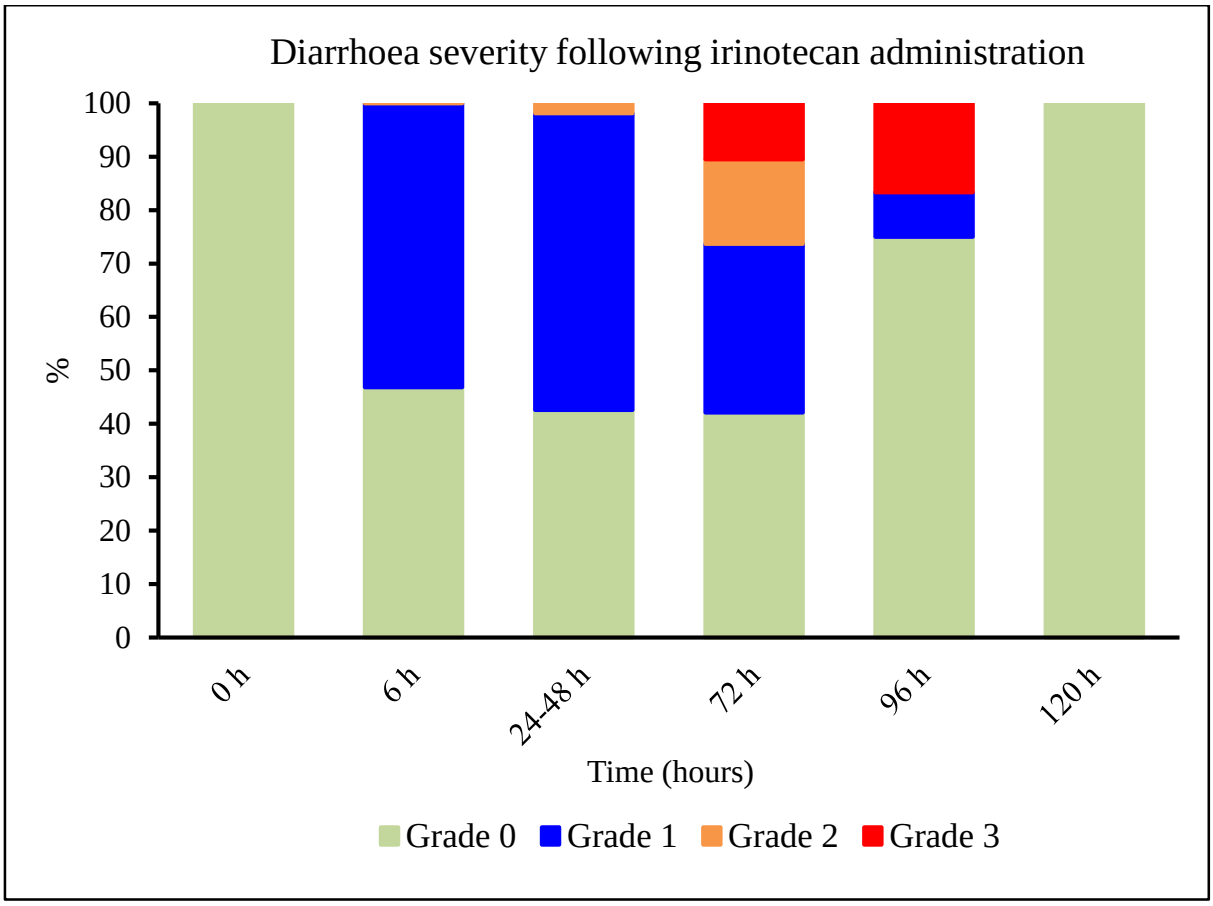

Figure 2.1. Incidence and severity of diarrhoea following irinotecan administration. Mild (grade 1), moderate (grade 2) and severe (grade 3) diarrhoea was observed between 6 and 96 h following irinotecan. Grade 3 diarrhoea was greatest between 72 h and 96 h. No diarrhoea (Grade 0) was observed in controls (0 h) and rats at 120 hours following irinotecan.

2.5.2 Irinotecan alters the composition of endogenous bacterial DNA in the caecum

The V3 region of bacterial 16s rRNA genes was analysed to determine levels of *Bifidobacterium* spp., *Lactobacillus* spp., *E. coli*, SRB and *Bacteroide*s spp. Levels of all bacteria except *Bacteroides* spp. were differentially altered by irinotecan (Figure 2.2). *Bifidobacterium* spp. was significantly decreased during times of greatest mucosal damage and diarrhoea, with levels at 96 h significantly lower compared to pre-irinotecan at 0 h (p=0.03) (Figure 2.2). Furthermore, at 48, 72 and 96 h, *Bifidobacterium* spp. levels were also significantly lower compared with those recorded at 120 h post-irinotecan (p=0.016, p=0.05 and p=0.02, respectively) (Figure 2.2). There was an 82% decrease in *Lactobacillus* spp. DNA levels at 96 h post irinotecan compared to *Lactobacillus* spp. DNA concentrations at 6 h (Figure 2.2). In contrast, *E. coli* concentrations were significantly increased at 72 h compared with 0 h (p=0.008) and 6 h (p=0.031. Levels of SRB were significantly increased at 96 h compared with 0 h (p=0.036) (Figure 2.2). *Bacteroides* spp. concentrations were slighted elevated but not significantly at 96 and 120 h post irinotecan administration compared to 0 h (Figure 2.2).

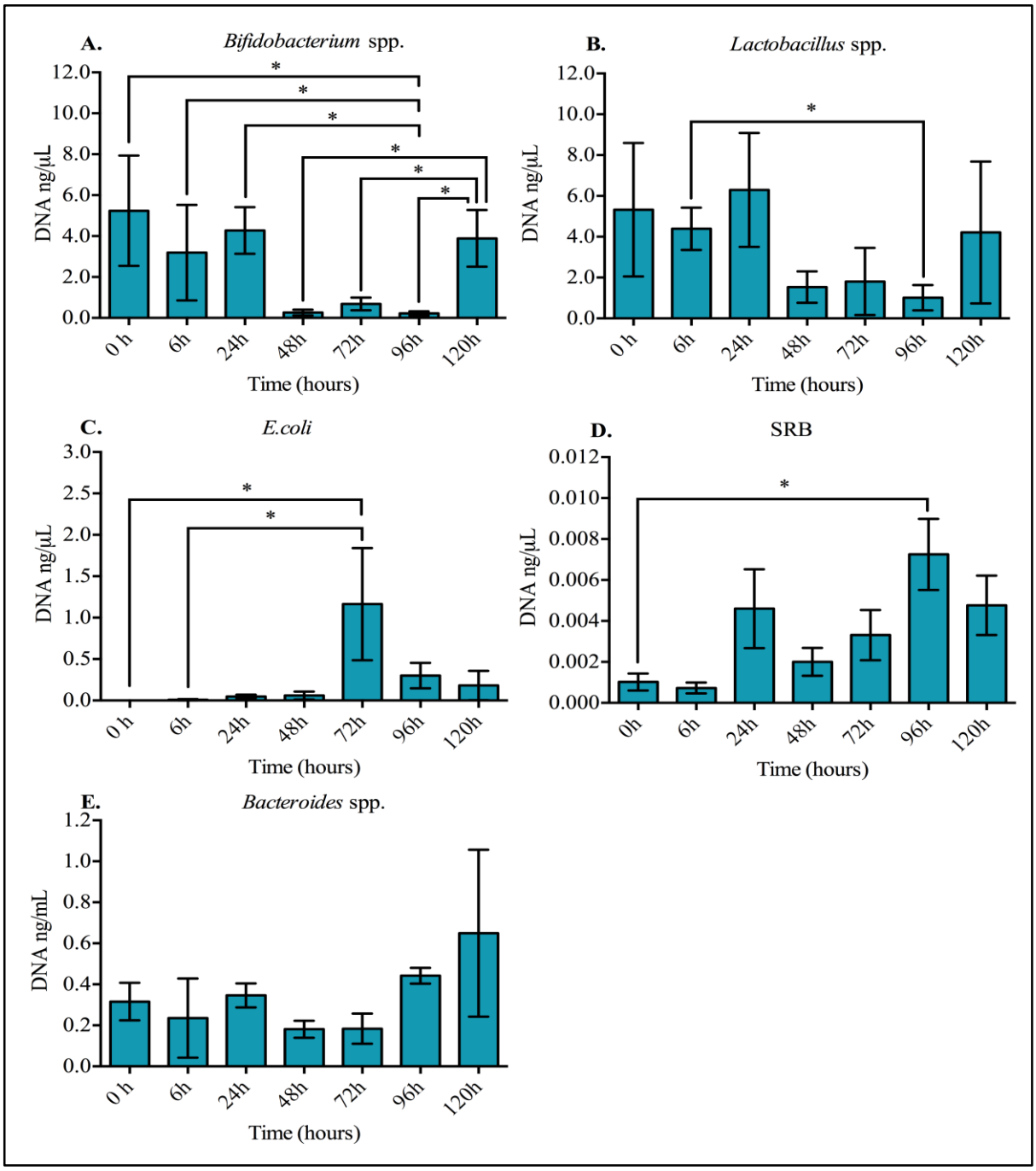

Figure 2.2. Bacterial DNA concentrations in the caecum following irinotecan administration. *Bifidobacterium* (A), and *Lactobacillus* spp. (B) levels significantly decreased, while *E. coli* (C), and SRB (D) levels were significantly increased following irinotecan administration. (E) *Bacteroides* spp. levels were raised but not significantly at 96 and 120 hours following irinotecan compared to controls. Values are expressed as mean ± SEM *p<0.05.

2.5.3 Irinotecan alters the composition of endogenous bacterial DNA in the colon

Significant changes were observed in concentrations of commensal bacteria and potentially harmful bacteria in colonic contents following irinotecan compared to controls. *Bifidobacterium* spp. levels were significantly decreased at 48 h (p=0.02) and 72 h (p=0.02) compared with levels at 6 h (Figure 2.3). *Lactobacillus* spp. concentrations decreased significantly at 72 h compared with rats at 6 h (p<0.004). Furthermore, concentration of *Lactobacillus* spp. DNA significantly increased at 120 h during the recovery phase compared to levels recorded in rats at 72 h (p=0.005) (Figure 2.3). *E. coli* levels were also significantly raised at 72 h compared with 0 h (p=0.004) (Figure 2.2). *E. coli* levels were significantly lower at 120 h, returning to baseline levels compared to levels at 72 h (p=0.038) (figure 2.3). There was a marginal elevation of *Bacteroides* spp. levels at 72 h post irinotecan during compared to controls but this was not significant (Figure 2.3).

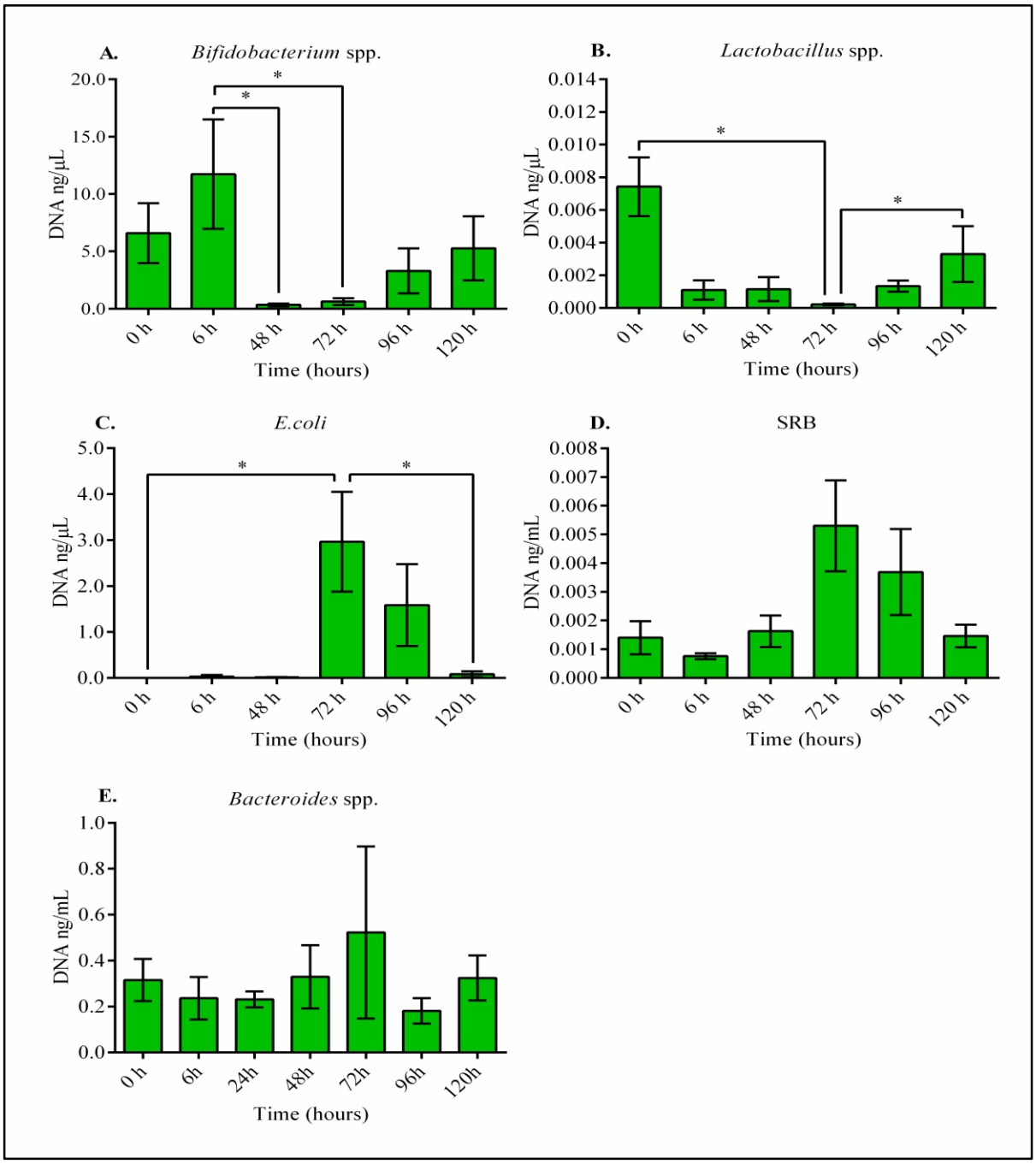

Figure 2.3. Bacterial DNA concentrations in the colon following irinotecan administration. *Bifidobacterium* spp. (**A**) and *Lactobacillus* spp. (**B**) levels were significantly decreased, while *E. coli* (**C**) and SRB (**D**) levels were significantly increased following irinotecan administration. (**E**) *Bacteroides* spp. levels were elevated at 72 h but not significantly compared to controls following irinotecan. Values are expressed as mean ± SEM; *p<0.05.

2.5.4 Irinotecan damages the mucosal surface in the jejunum of tumour-bearing DA rats

Irinotecan induced structural changes in the GIT. Villus fusion and villous blunting increased between 24 and 72 h following irinotecan administration compared with rats receiving no irinotecan (0 h). Histological repair in the GIT was evident at 120 h following treatment (Figure 2.4).

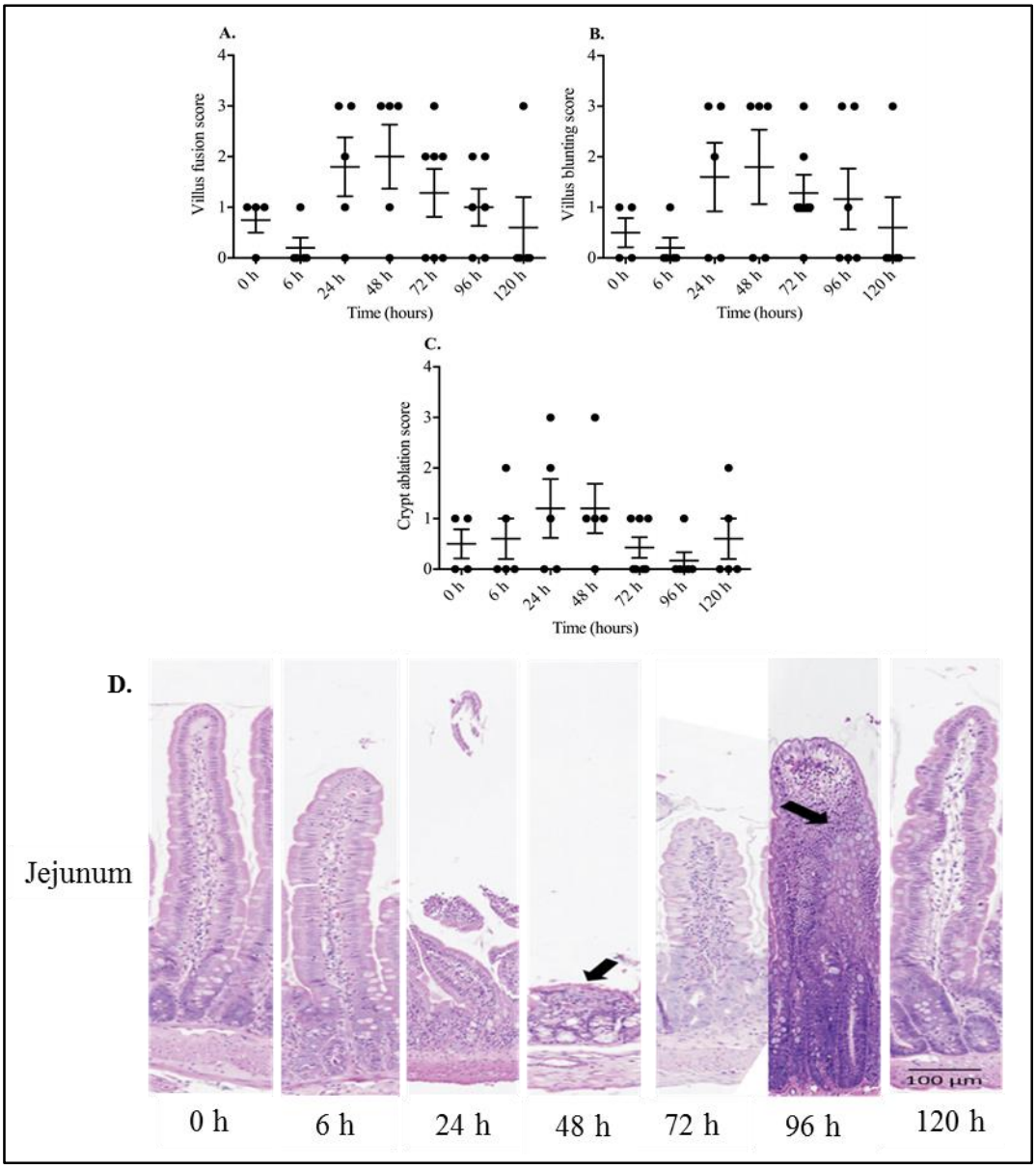

Figure 2.4. Irinotecan damages the mucosal architecture in the jejunum following irinotecan administration. Greatest damage occurred between 24 h and 96 h after irinotecan administration. Parameters include (**A**) Villus fusion, (**B**) Villus blunting and (**C**) Crypt ablation. **D**) H&E images show changes in the mucosal architecture (arrows) observed at 48 and 96 h following irinotecan. At 120 h following irinotecan injection, villus height returned to control levels. Values are expressed as mean ± SEM

2.5.5 Irinotecan causes crypts ablation in the colon of tumour-bearing DA rats.

Crypt ablation was significantly increased in the colon compared to controls at 24, 72 h and 96 h following irinotecan (Figure 2.5). Crypt ablation appeared to decrease during the recovery phase at 120 h.

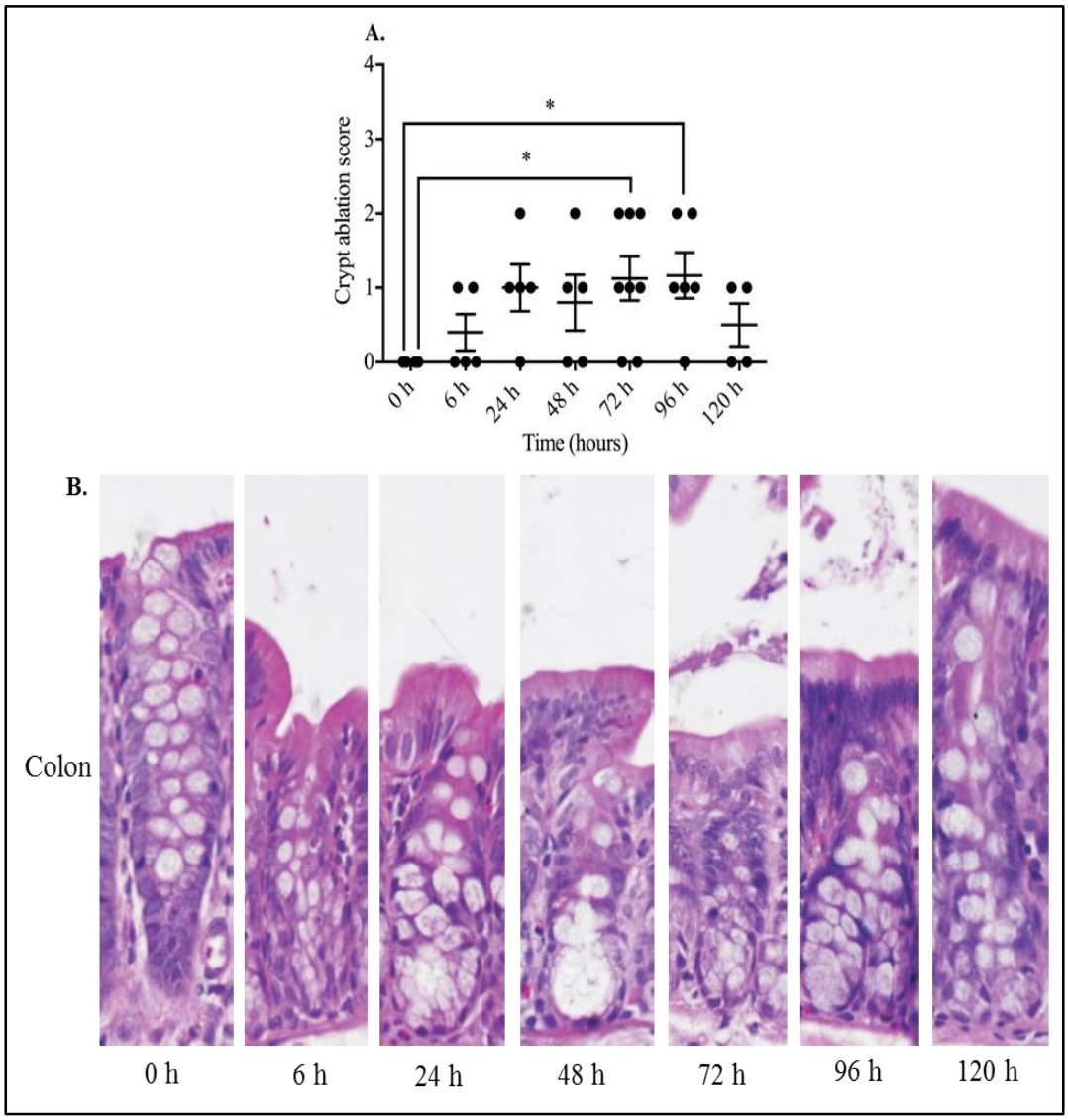

Figure 2.5. Irinotecan changes the mucosal architecture in the colon following irinotecan administration. (**A**) Crypt ablation was significantly higher at 24 h, 72 h and 96 h, following irinotecan administration. (**B**) In the colon crypt ablation (arrows) was evident at 72 h following irinotecan. Values are expressed as mean ± SEM. *p<0.05.

2.5.6 *Bacterial changes are associated with colonic damage and diarrhoea resulting from irinotecan administration.*

There was a significant association between colonic *E. coli* concentrations and diarrhoea score at 72 h and 96 h post-irinotecan (r=0.76 (95% CI 0.2947 to 0.0916), p=0.01) (Figure 2.6). There were no other associations found between microbial populations and other parameters of chemotherapy-induced mucositis.

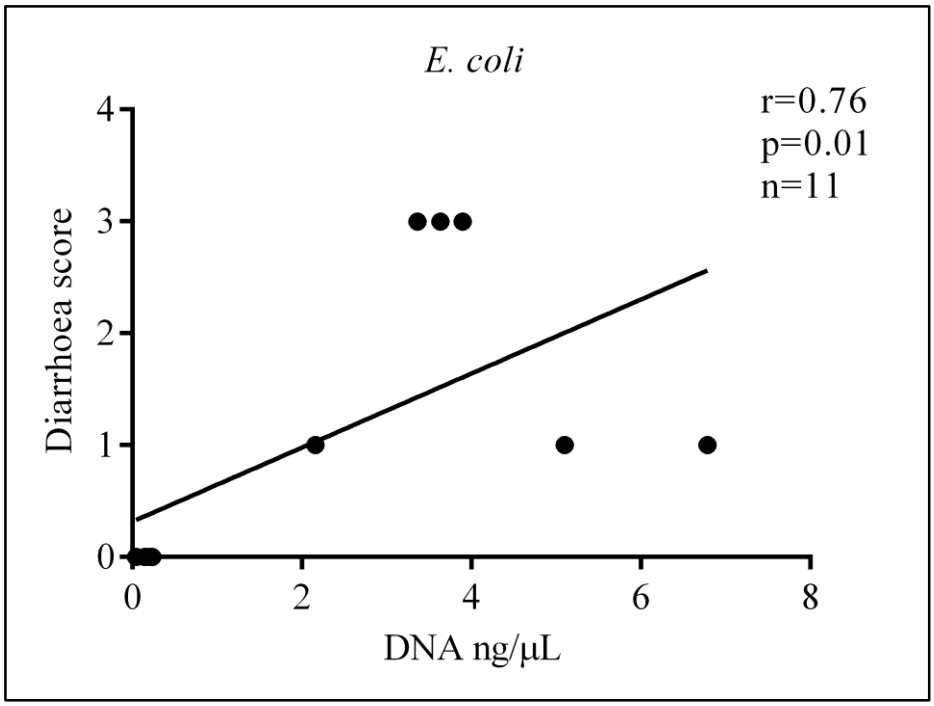

Figure 2.6. *E. coli* levels and diarrhoea score following irinotecan. Increased *E. coli* levels in the colon were significantly correlated with diarrhoea score between 72 and 96 h following irinotecan.

2.6 Discussion

Intestinal mucositis caused by chemotherapeutic agents is debilitating, sometimes life threatening, and clinically difficult to manage (Lalla, Bowen, Barasch *et al.* 2014). The emerging importance of the microbiome in the pathogenesis of intestinal mucositis represents potential new strategies to personalise chemotherapy regimens to minimise associated toxicities. This study identified a decrease in mucosally beneficial bacteria (*Lactobacillus* and *Bifidobacterium* spp.) and a concomitant increase in potentially pathogenic *E. coli* and SRB in the caecum and colon of rats with irinotecan induced mucositis. This was associated with histological damage in the jejunum and colon, and diarrhoea score. It is important to note that all rats in this study were tumour bearing and may have an altered baseline microbiome compared with healthy non-tumour bearing rats, due to a baseline immunological response inherently altering the microbiome. In any case, these results suggest that decreased levels of beneficial bacteria and increased concentrations of pathogenic bacteria maybe a contributing factor for diarrhoea severity induced by chemotherapy in this model.

Changes appear to occur at the time of greatest damage to the small intestinal and colonic mucosa, which relate to the most severe diarrhoea, corresponding to times of maximal mucosal stress. Irinotecan has been shown to affect the composition of the intestinal microbiota, favouring bacteria with β-glucuronidase activity, which may increase the toxicity of irinotecan in the GIT (Stringer, Gibson, Logan *et al.* 2009, Zwielehner, Lassl, Hippe *et al.* 2011, Montassier, Batard, Massart *et al*. 2014). This may increase mucosal stress leading to increased intestinal permeability and, disrupted barrier function exposing

immune cells embedded in the underlying tissue to a vast array of metabolites and microorganisms in the GIT (Davidson, Kritas and Butler 2007). This may trigger an inflammatory cascade in the mucosa, already under stress from cytotoxic agents. *Lactobacillus* spp. exhibits anti-β-glucuronidase activity which decrease or decrease the hydrolysis of SN-38G to SN-38 in the GIT (Sreekumar and Hosono 2000).

In vitro, *L. acidophilus* (SBT 2074) cell free concentrate showed effectiveness at inhibiting a range of Gram-positive and Gram–negative bacteria such as *Listeria monocytogenes*, *Bacillus subtilis*, and *Enterobacter aerogenes* (Sreekumar and Hosono 2000). In co-culture with *E. coli*, *L. acidophilus* (SBT 2074) significantly decreased growth rates of *E. coli* (Sreekumar and Hosono 2000). *In vivo* studies have also shown that rats given *L. acidophilus* (SBT 2074) had significantly lower counts of coliforms and significantly lower levels of β-glucuronidase activity tested in faeces compared to control rats not treated with *L. acidophilus.* The significantly decreased levels of β-glucuronidase activity could be directly related to the decreased numbers of *E. coli* (Sreekumar and Hosono 2000). Therefore, the significant decrease of *Lactobacillus* spp. observed at times of greatest mucosal stress may have an impact on the damage caused by the potential increase in bacteria with β-glucuronidase leading to an increase in the toxic metabolite SN-38.

Lactobacillus and *Bifidobacterium* spp. are known beneficial microbes positively influencing the GIT mucosa (Turroni, Ventura, Buttó *et al.* 2014). Irinotecan disrupts DNA replication in rapidly dividing cells, disrupting intestinal epithelial cell regeneration affecting mucosal structure and function. Mucosal damage concomitantly occurring with

decreased numbers of *Lactobacillus* and *Bifidobacterium* spp. may directly decrease the competitive advantage of these beneficial microorganisms. This may allow adherent and invasive *E. coli* to proliferate under the altered gastrointestinal environment. *E. coli* is known to preferentially colonize damaged mucosae. Biopsies from ulcerative colitis (UC) and Crohn's disease (CD), patients where stressed mucosa is observed show levels of *E. coli* are increased compared with healthy controls (De Souza, De Carvalho, Romeiro *et al.* 2012). *E. coli* have pili and flagella allowing adherence to host cells through adhesion carcinoembryonic antigen–related cell adhesion molecule 6 (CEACAM6), which is over-expressed in CD patients compared to healthy controls. CEACAM 6 can also be up-regulated by adherent and invasive *E. coli* providing a competitive advantage (Barnich, Carvalho, Glasser *et al.* 2007). Increased levels of *E. coli* and decreased levels of beneficial bacteria at times of greatest mucosal stress may promote an environment of inflammation and damage, further enhancing the damage caused by irinotecan.

Both *in vitro* and *in vivo* studies have demonstrated that *Lactobacillus* and *Bifidobacterium* spp. can inhibit adhesion of pathogenic organisms to the mucosa (Bernet, Brassart, Neeser *et al*. 1993, Lievin, Peiffer, Hudault *et al*. 2000, Johnson- Henry, Hagen, Gordonpour *et al.* 2007, Liévin-Le Moal and Servin 2014). *In vitro* studies have shown that *Bifidobacterium* spp. have a powerful antagonistic effect against the pathogens *E. coli* and *Clostridium perfringens.* Batch fermenters were inoculated with *B. infantis*, *E. coli* and *C. perfringens* and grown for 60 h. Bacterial levels were taken at various time points and showed significantly reduced counts of *E. coli* and *C. perfringens* with levels of *B. infantis* remaining stable (Gibson and Wang 1994). Furthermore, the supernatant of *B.*

infantis was also able to inhibit the growth of these pathogenic bacteria (Gibson and Wang 1994). Others have shown that *B. infantis* Y1 given to mice was able to up regulate the production of tight junction proteins decreasing transmembrane resistance in colonic segments in Ussing chambers (Ewaschuk, Diaz, Meddings *et al.* 2008). Significantly decreased levels of *Bifidobacterium* spp. observed in this study may therefore be deleterious to the maintenance of intestinal permeability and promote stressed mucosa.

In other studies Bifidobacterial adhesin, a protein secreted by *B. adolescentis* was shown to aid mucosal repair in a rat model of stress, by decreasing the levels of pro-inflammatory cytokines, NFκB and TNF-α in which rats were confined for 6 h per day for 21 days to induce stress (Shu, Yu, Kang *et al*. 2014). This, combined with the anti β-glucuronidase activity of *Lactobacillus* spp. suggests that these beneficial bacteria may be mucosally protective during chemotherapy. In a recent study investigating the microbiome of patients undergoing chemotherapy the authors reported decreased levels of the mucosally protective *Bifidobacterium* spp. and *F. prausnitzii*, with increased levels of Proteobacteria (*E. coli*) (Montassier, Gastinne, Vangay *et al.* 2015). Decreases in beneficial bacteria in this study were also related to an inability to modulate the effects of the pro-inflammatory cytokines NFκB and TNF-α (Montassier, Gastinne, Vangay *et al.* 2015). These compositional shifts in the microbiome of chemotherapy patients appear to promote a pro inflammatory environment leading to mucositis, suggesting a causal role of the microbiome in the development of chemotherapy-induced mucositis (Montassier, Gastinne, Vangay *et al*. 2015). In the current study an imbalance in the microbial composition was detected favouring increased *E. coli* and decreased *Bifidobacterium* and

Lactobacillus spp. This imbalance may have contributed to mucosal damage following irinotecan. Interestingly, during the recovery phase at 120 h, the imbalance in microbial populations again shifted towards a more beneficial/mucosally protective pattern.

SRBs have not previously been investigated in a chemotherapy-induced mucositis setting. SRBs are commensal microbes of the GIT that utilize the reducing power of hydrogen produced by the microbiota to produce hydrogen sulphide gas (H_2S) (Croix, Carbonero, Nava *et al.* 2011). H_2S is a mucosal irritant and genotoxic, and has been implicated in contributing to inflammatory conditions of the colon, such as UC and pouchitis (Levine, Ellis, Furne *et al.* 1998, Ohge, Furne, Springfield *et al*. 2005). Secretion of sulphated mucins is increased at 72 h post irinotecan administration in rat model of chemotherapy-induced mucositis (Stringer, Gibson, Logan *et al*. 2009). Levels of NFκB and TNF-α rise during mucosal damaged (Majuri, Niemelä, Tiisala *et al.* 1995) and can also be increased by non-pathogenic *E. coli* (Borruel, Casellas, Antolín *et al.* 2003). NFκB and TNF-α increase the expression of glycosyltransferases and sulfotransferases responsible for the biosynthesis of sulphated mucins (Majuri, Niemelä, Tiisala *et al*. 1995). Raised levels of sulphated mucins may act as a substrate for SRBs, elevating H_2S levels, further contributing to the mucosal irritation during chemotherapy. Interestingly, *Bacteroides* spp. concentrations were elevated in the colon at 72 h post irinotecan compared to controls and corresponded with the increased colonic SRB levels at 72 h. *Bacteroides* spp., specifically *B. fragilis*, has sulfatase activity and is able to liberate sulphate from endogenous mucins which can be subsequently used by SRB for sulphate reduction (Willis, Cummings, Neale *et al.* 1996). *Bacteroides* spp. may be a potential

organism to target in future studies as they may indirectly increase sulphate reduction by liberating sulphate from sulphated mucins during chemotherapy. Furthermore, studies have also shown that SRB preferentially utilize sulphate from sulphated mucins rather than those derived from the diet (Deplancke, Finster, Graham *et al.* 2003). Another interesting finding in the recently published study by Montassier *et al* 2015, was that sulphur metabolism was increased and that this may have accounted for increased oxidative stress following chemotherapy (Montassier, Gastinne, Vangay *et al.* 2015). This suggests that increases in SRB reflected in this study may be influenced by the known increase in sulphated mucins during irinotecan-induced mucositis, and specific *Bacteroides* spp. with sulfatase activity, subsequently increasing levels H_2S. A clearer understanding of SRB and the potential role they play in chemotherapy-induced mucositis is warranted.

In this study, a decrease in beneficial bacteria was observed, and inversely, an increase in pathogenic species occurred, correlating with increased mucosal damage and diarrhoea. This has been shown in other models of chemotherapy-induced mucositis (Stringer, Gibson, Logan *et al.* 2008). However, while this study did not aim to associate microbiome changes with diarrhoea, there appears to be similar association between decreased *Bifidobacterium* spp. and raised levels of *E. coli* during diarrhoea. Interestingly, there was an abrupt change in the amount of *E. coli*, SRB, *Bifidobacterium* spp. and *Lactobacillus* spp. detected at 72 h compared to levels recorded at 120 h, which corresponded to the mucosa returning to a pre-irinotecan state. This suggests that the ratio of beneficial to potentially pathogenic bacteria is important to understand in the

context of the time course of chemotherapy. These patterns provide a potential course of action, by which the microbiota can be altered or bolstered pre-treatment (by prebiotics or probiotics) or at specific times during the course of treatment to ameliorate symptoms of mucosal damage.

In previous studies of chemotherapy-induced mucositis investigators have used faeces for analysis of changes to GIT microbial populations following irinotecan treatment (Stringer, Gibson, Logan *et al*. 2008). While this gives information in a general way about the bacteria present in the GIT, this method of faecal collection may not be completely representative of changes happening at times of greatest damage and healing in the different geographical niches of the GIT. In this study, caecal and colonic contents were analysed using qPCR and show marked regional variations between irinotecan-treated and control rats over the 120 h time course. Moreover, levels of all bacteria tested over the time course were different in the different geographical niches tested. This seems to imply that potential treatment regimens need to use regionally specific bacteria to elicit maximal protective effects in the different geographical niches throughout the GIT during chemotherapy.

Finally, by understanding how the shifts occur over the time course of chemotherapy we can implement strategies to redress or prevent these detrimental compositional shifts that appear to be detrimental from occurring. These findings will allow us to develop new experiments that enable priming of the microbiome pre-treatment (to promote an environment which is mucosally protective), and to target the timing of potential probiotic

administration at times of greatest damage. The main findings of this study were that a potential pathogen and a putative pathogenic bacterium were raised at times of greatest mucosal damage and corresponded to diarrhoeal severity. This was occurring at times when endogenous beneficial bacteria were significantly reduced, promoting an environment conducive to proliferation of *E. coli* and SRB. Further studies investigating strategies to selectively augment the populations and activity of the beneficial bacteria in a patient-specific manner are required, involving characterising endogenously beneficial microbes, and selecting for increased adherence to the mucosa under stress.

2.7 Conclusion

In conclusion, decreased levels of the endogenously beneficial bacteria (*Lactobacillus* and *Bifidobacterium* spp.) and increased putative pathogens (*E. coli* and SRB) may modulate the severity of damage and recovery time frame following irinotecan, suggesting the use of this type of data as a biomarker to indicate severity of damage, and also repair to the mucosa following irinotecan. Understanding changes in the endogenous commensal microorganisms following chemotherapy is likely to play a role in defining a patient-specific approach to the treatment of mucositis based on the endogenous microbiota present prior to treatment.

Chapter 3.0 Adaptive patterns in the intestinal microbiome from virally induced gastroenteritis in Australian children

3.1 Preface

In this chapter it was observed that compositional shifts in the microbiome occurred in response to two different viral infections compared to healthy controls. Acute gastroenteritis (AGE) results in short term mucosal stress, with the growing belief that changes in the mucosal barrier accompanied by compositional shifts in the microbiome may affect disease susceptibility in some individuals in the future. Compositional shifts in the microbiome may affect the integrity of the mucosal barrier under stress, resulting in permeability changes that may have adverse effects including severity of diarrhoea. Viral infections cause hundreds of millions of cases of AGE annually with the full impact widely underestimated. This study aimed to investigate selected bacterial changes in the microbiome in two different viral infections and compared these to healthy controls, with the aim of assessing ways to alleviate symptom severity in the future during AGE. The microbiota was altered in both viral infections and the microbiome showed different adaptive patterns between the two viruses. Levels of the endogenous beneficial *Bifidobacterium* spp. correlated with decreased bowel frequency in rotavirus (RV) infection suggesting that elevated levels of these microbes may ameliorate symptoms.

3.2 Abstract

Introduction

Rotavirus (RV) is a major cause of acute gastroenteritis (AGE) worldwide resulting in both morbidity and mortality. A paucity of information exists regarding the effect of RV & human boca virus 2 (HBoV2) induced AGE on the balance of endogenous probiotics & commensal bacteria within the microbiome.

Material and Methods

Faecal samples from children with two distinct viral infections (RV, n=32 and HBoV2,n=32) were analysed using PCR, and compared with healthy age matched controls(n=32).

Results

Higher levels of *Bifidobacterium* spp. in RV infection were associated with decreased frequency of bowel movements ($r=0.42$, $p<0.02$). In RV infection *Lactobacillus* spp. were elevated ($p<0.03$) compared to controls but no relationship with the severity of diarrhea was seen. *Bifidobacterium* spp. levels were not significantly different to healthy control subjects. *Enterococcus* spp. ($p<0.05$) and *E. coli* ($p<0.06$) levels were not statistically different compared to controls, nor were *Bacteroides* spp. levels significantly different to controls. In HBoV2 infection *Lactobacillus* spp. were elevated ($p<0.02$) compared with controls, *Enterococcus* spp. ($p<0.003$) and *E. coli* levels ($p<0.005$) were elevated compared to controls. *Bifidobacterium* spp. levels were significantly higher in RV infection compared with HBoV2 infection ($p<0.04$). The potentially pathogenic *E. coli* were elevated in HBoV2 infection compared with levels in RV infection. The relative levels of *Bifidobacterium* spp. compared with *E. coli* levels were significantly higher in RV infection compared with HBoV2 infection.

Conclusions

This study has shown that the microbiome was differentially altered in two viral gastrointestinal infections. This data implies distinct mechanisms of induction of diarrhoeal disease resulting in selective adaptive responses of the microbiome. Opportunistic pathogens such as *E. coli* may be increased in RV and HBoV2 infection. Alternatively the levels of protective species may aid in modulating the severity of diarrhoeal disease. Larger studies are now required to confirm whether these observations can inform us of favourable microbiome modifying interventions.

3.3 Introduction

A stressed mucosa can arise from a wide range of external and internal factors, including viral infections (Davidson, Kritas and Butler 2007). This is particularly common during infancy and early childhood and can cause a stressed mucosa leading to severe diarrhoea which can be life threatening. Moreover, in developing countries viral diarrhoea accounts for over 500, 000 deaths per year (De Oliveira, Giglio, Ciapponi *et al.* 2013). AGE induced as a consequence of viral infections may disrupt the fine balance of microorganisms in the GIT. Changes to the microbiome in infancy has been proposed as an instigator of further complications later in life that have no clear aetiological precursors including asthma, atopic eczema, hayfever, inflammatory bowel disease and irritable bowel syndrome (Isolauri 2012, Cremon, Stanghellini, Pallotti *et al*. 2014). As the largest cellular interface between the outside environment and the internal milieu, the mucosa and its interaction with the microbiome (where maturational plasticity is thought to exist during early childhood) is crucial for balanced immune system interactions, and programming during the early developmental period of infancy and early childhood. A paucity of information exists concerning RV, a common cause of AGE in childhood, and HBoV2, a newly discovered virus associated with childhood diarrhoea, and the relationship with endogenous beneficial bacteria, opportunistic pathogens and a large commensal genus within the faecal microbiome during infection.

RV infection is the most common cause of childhood AGE worldwide causing up to 40% of all diarrhoea cases (Bishop 2009). Despite advances in RV vaccines and decreased cases of RV-associated diarrhoea (in developed countries), intussusception (a life

threatening side effect) still occurs. Intussusception is a condition in which part of the intestine invaginates itself into another section of the intestine, and is associated with the RV vaccination (Fischer, Bihrmann, Perch *et al*. 2004, Weintraub, Baggs, Duffy *et al.* 2014). It has been reported that there is an increased risk of intussusception in infants receiving the 2nd dose of the monovalent vaccine (Rotarix), but not the pentavalent vaccine (RotaTeq) (Weintraub, Baggs, Duffy *et al.* 2014). However, despite the introduction of vaccines in developed nations, RV is still a major cause of mortality and morbidity in developing countries in children under five years of age (Boshuizen, Reimerink, Korteland-Van Male *et al.* 2005, Kirkwood, Boniface, Bishop *et al.* 2009). Recently, HBoV2 has been associated with diarrhoeal disease in children, and may account for up to 15% of diarrhoeal illness in this cohort (Arthur, Higgins, Davidson *et al.* 2009). Little is known about the microbial changes in the gastrointestinal microbiome during RV and HBoV2 infections in paediatric patients.

3.3.1 Rotavirus

RV has several serotypes, A through to E with serotype A being the most common to infect humans. Serotype A is classified into four main subgroups based on antigenic determinants on the outer capsid (Lundgren and Svensson 2001, Hyser and Estes 2009). RV is a naked double stranded RNA virus transmitted by human-to-human contact. Symptoms of RV infection are, mild to severe diarrhoea, mild vomiting, headache and fever. RV is self-limiting, with duration of illness between 1 and 5 days. Children younger than five years of age are most susceptible to RV infection. The elderly are also at higher risk of RV infection, and mortality may be as high as 50 % in those over 73 years (Arthur, Higgins, Davidson *et al*. 2009).

The apical cells of the duodenum and jejunum are severely affected by RV, causing necrosis and leading to malabsorption and profuse watery diarrhoea, with concomitant loss of electrolytes and solutes causing dehydration (Ramig 2004). RV also causes diarrhoea by secretion of non-structural protein (NSP) 4, which acts as a toxin stimulating the enteric nervous system, which may contribute up to 60% of total water loss seen in RV diarrhoea (Ramig 2004). Additionally, NSP 4 leads to disruptions in tight gap junctions of the GIT that ultimately leads to electrolyte changes in the intestine culminating in diarrhoea (Ramig 2004).

3.3.2 Human boca virus 2

HBoV2 is a parvovirus, belonging to the subfamily Parvovirinae. HBoV2 has been associated with AGE in a paediatric cohort. HBoV2 is yet to be confirmed as a potential pathogen causing diarrhoea. There is no information currently available on how HBoV2 affects the gastrointestinal mucosa and the mechanism of action in contrast to the well documented pathogenesis of RV infection (Ramig 2004).

3.3.3 Mucosal stress during acute gastroenteritis

RV produces NSP 4, which stimulates the enteric nervous system and leads to villous necrosis and is confined to the small intestine (duodenum and jejunum). The blunting of the small intestinal villi disrupts the apical junctions between cells leading to increased permeability, severe inflammation and mucosal stress (Ritchie, Brewster, Davidson *et al.* 2009). As the small intestinal mucosa is severely affected during RV, the protective effect of the microbiome and barrier function is altered. This exposes the underlying immune

cells to foreign antigens initiating a cascade of responses leading to immune system activation that may persist long after the initial infection has resolved. The mucosal interface and the microbiome have an intimate relationship needed for appropriate immune system development (Maynard, Elson, Hatton *et al.* 2012). Mice grown under germ-free conditions show decreased growth, increased risk of infections, need supplemented vitamins and minerals, and show changes in gut morphology and physiology compared with mice grown with a diverse intestinal microbiome (Alam, Midtvedt and Uribe 1994). Decreased species diversity and abundance may also arise due to the infection, with the microbiome unable to restore itself to its pre-infected state. If the microbiome is unable to recover to the pre-existing microbial composition existing prior to infection, this may trigger an aberrant immune response leading to illness. Alternatively, increased susceptibility to illnesses post infectious gastroenteritis may arise as a consequence of a disrupted intestinal mucosal barrier unable to reorganise itself after the initial infection, exposing the underlying immune system to further insult (Piche 2014).

3.4 Aims

The aims of this study were to quantitate changes in selected endogenous beneficial bacteria, potential pathogens and a large commensal genus in the intestinal microbiome in a paediatric population during two viral infections associated with diarrhoea and compared to a healthy age matched control group.

3.5 Materials and Methods

This study quantitated the faecal microbiota in stool samples of children with RV (n=32) and HBoV2 infection (n=32) compared to healthy age matched controls (n=32). Two endogenous beneficial species (*Bifidobacterium* and *Lactobacillus* spp.), a large commensal genus (*Bacteroides* spp.), and two potentially pathogenic commensal bacteria (*E. coli* and *Enterococcus* spp.), were quantitated using real time PCR.

3.5.1 Sample collection and processing

Archived faecal samples from a study published by Arthur *et al,* (2009), performed at the Women's and Children's Hospital (Adelaide) to investigate the aetiology of AGE in paediatric patients, were used for this research (n=96). Arthur *et al*, reported a newly identified virus, HBoV2 and this was statistically associated with diarrhoeal illness in these patients (n=32). Routine screening of causal pathogens (microscopy and culture) (*Salmonella* spp., *E. coli*), of parasites (Giardia; microscopy) and other viral agents (rotavirus, calicivirus, astrovirus, adenovirus and, sapovirus) was also undertaken in all patients presenting with AGE in the above study with RV determined to be the causative agent in selected patients (n=32) (Arthur, Higgins, Davidson *et al.* 2009). No routine screening was carried out on healthy controls. Healthy controls (n=32) comprised hospital patients or siblings of patients, all of whom were free from any gastrointestinal symptoms and antibiotic use, two weeks prior to sample collection. Ethics approval was obtained from the University of South Australia, Institute for Medical and Veterinary Science (IMVS) and The Women's and Children's Hospital Human Research Ethics Committees. Faecal samples were stored at -70°C at the IMVS Adelaide, South Australia.

3.5.2 Extraction of total DNA

Bacterial DNA was extracted from 0.1 g of faeces using the bead beating plus column method described by Yu and Morrison (Yu and Morrison 2004). Briefly, faeces were thawed, then homogenised with garnet beads (0.70 mm) in lysis buffer (29.22 g/L NaCl, 6.075 g/L Tris base, 18.612 g/L ethylenediaminetetraacetic acid [EDTA], and 40 g/L sodium dodecyl sulfate [SDS], all purchased from Sigma Aldrich, in milliQ water, pH 8). Samples were then incubated at 70°C for 15 min. Precipitation of nucleic acid was achieved with a set of incubation and centrifuging steps using ammonium acetate, isopropanol and 70% ethanol. Nucleic acid pellet was dissolved in Tris-EDTA (TE) buffer (12.1 g/L Tris base and 3.72 g/L EDTA in milliQ water, pH 8). Total DNA concentration was determined using a Nanodrop 2000 (Thermo-Fisher Scientific, Scoresby, Australia). DNA was diluted to a working concentration of 5 ng/µL. DNA levels were determined using a Nano Drop spectrophotometer 2000 (Thermo Fisher Scientific, Scoresby, Australia). Total DNA concentration and purity (260/280 nm) were determined using a Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, Scoresby, Australia). Each sample was diluted to a working concentration of 5 ng/µL.

3.5.3 Quantitative real-time PCR

Real-Time PCR analysis of faecal samples was performed to quantify levels in the RV, HBoV2 and control groups using the V3 region of the 16S rRNA genes of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp., *E. coli* and *Enterococcus* spp., representing endogenous beneficial commensals and opportunistic pathogens. All primers in this study have been used previously (Table 3.1). Real-Time PCR was carried out on a Rotor Gene-Q rotary cycler (QIAGEN, Australia), using Quantitect® SYBR green mastermix (Qiagen).

Briefly, each reaction consisted of 5µL of 2x Quantitect® SYBR green mastermix (Qiagen), 2.5 ng/µL of each primer (forward and reverse (3 µL total) and 10 ng (2 µL total) of sample in a volume of 10 µL. Each sample was tested in triplicate. Standard curves were constructed using 5 serial dilutions of known bacterial DNA. Data was analysed using Rotor Gene Q-software (QIAGEN, Australia).

Table 3-1: Primers used for Real-Time PCR.

Target Organism	Primer	Primer sequence (5' - 3')	Size (Bp)
Bifidobacterium spp. (Rinttila *et al*.2004)	Bifido R Bifido F	CAC CCG TTT CCA GGA GCT ATT GCG TGG TTA ACA CAT GCA AGT C	243
Bacteroides spp. (Layton *et al*.2006)	Bac R Bac F	GAG AGG GTC CCC CAC CGC TAC TTG GCT GGT TCA G	106
Lactobacillus spp. (Rinttila *et al*.2004)	Lacto R Lacto F	CAC CGC TAC ACA TGG AG AGC AGT AGG GAA TCT TCC A	341
E. coli (Penders *et al*.2005)	E. coli R E. coli F	CGC GTA ACG TCA ATG AGG AAA CAT GCC GCG TGT ATG AAG AA	95
Enterococcus spp. (Rinttila *et al*.2004)	Entero R Entero F	ACT CGT TGT ACT TCC CAT TGT CCC TTA TTG GTT GCC ATC ATT	144

3.5.4 Statistical analysis

Statistical analysis of data comparing levels of selected bacteria quantitated from faecal samples using PCR from the RV, HBoV2 and healthy control groups was carried out using Graphpad Prism 6 software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). Pearson's correlation and Spearman's correlation were used to correlate changes between intestinal parameters of mucosal stress and faecal levels of selected bacteria. Normality of data was tested using D'Agostino & Pearson omnibus normality test. Non-parametric data were analysed using the Mann Whitney U test, with Bonferroni test used to determine significance of p values. $p < 0.05$ was considered as significant. Data are expressed as mean ± SEM.

3.6 Results

The present study analysed faecal samples (n=96) of three well-defined groups as follows: RV infection (n=32), HBoV2 infection (n=32) and aged matched healthy controls (n=32) (Table 3.2). In both viral infection groups there was only one causative agent detected, either RV or HBoV2. Number of bowel movements in a 24 h period was recorded in both diarrhoeal groups (Table 3.3).

Table 3-2: Mean age in years of subjects in the rotavirus group, human boca virus 2 group and healthy controls.

Groups	Age in years	Mean age in years	Total number
Human boca virus 2	0.1–9.9	1.3	32
Rotavirus	0.7–5.4	1.8	32
Controls	0.1–9.4	2.2	32

Table 3-3: Frequency of bowel movements in rotavirus and human boca virus 2 groups over a 24-hour period.

Groups	Total number of bowel movements over 24 hrs	Mean number of bowel movements in a 24 h period (±SD)
Human boca virus 2	3-25	8.04 ± 6.62
Rotavirus	3-12	6.38 ± 2.54

3.6.1 Microbiota changes in rotavirus infection

Higher *Bifidobacterium* spp. levels were correlated (r=-0.42 (95% CI: -0.68 to -0.08), p=0.02) with decreased frequency of bowel motions in a 24 h period in RV infection (Figure 3.1). *Lactobacillus* spp. DNA levels were significantly raised in RV infection (0.42 ± 0.14 ng/µL) compared to control levels (0.19 ± 0.04 ng/µL) (p=0.03; Figure 3.2). *E. coli* concentrations were significantly raised in RV infection compared with healthy controls (p=0.04) (Figure 3.2). Levels of *Enterococcus* spp. were elevated compared with controls (p=0.07) (Figure 3.2). *Bifidobacterium* spp. levels were not significantly different to controls (p=0.13; Figure 3.2). The large commensal group *Bacteroides* spp. DNA were not altered compared to controls levels in RV infection (Figure 3.2). Concentrations of *Bifidobacterium* spp. DNA were raised compared to *E. coli* DNA levels in RV infection (Figure 3.2).

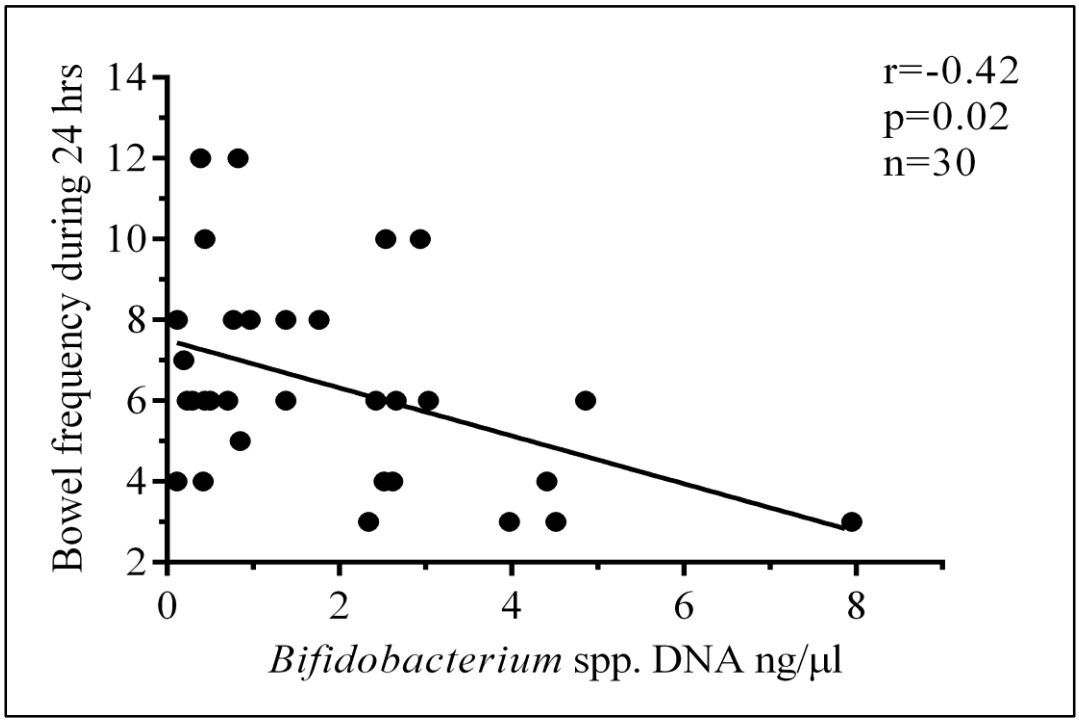

Figure 3.1. Faecal concentrations of *Bifidobacterium* spp. DNA in rotavirus infection compared with frequency of bowel movements over 24 h. *Bifidobacterium* spp. levels were significantly correlated with decreased bowel frequency during RV infection (r=-0.42 (95% CI: -0.68 to -0.08); p=0.02).

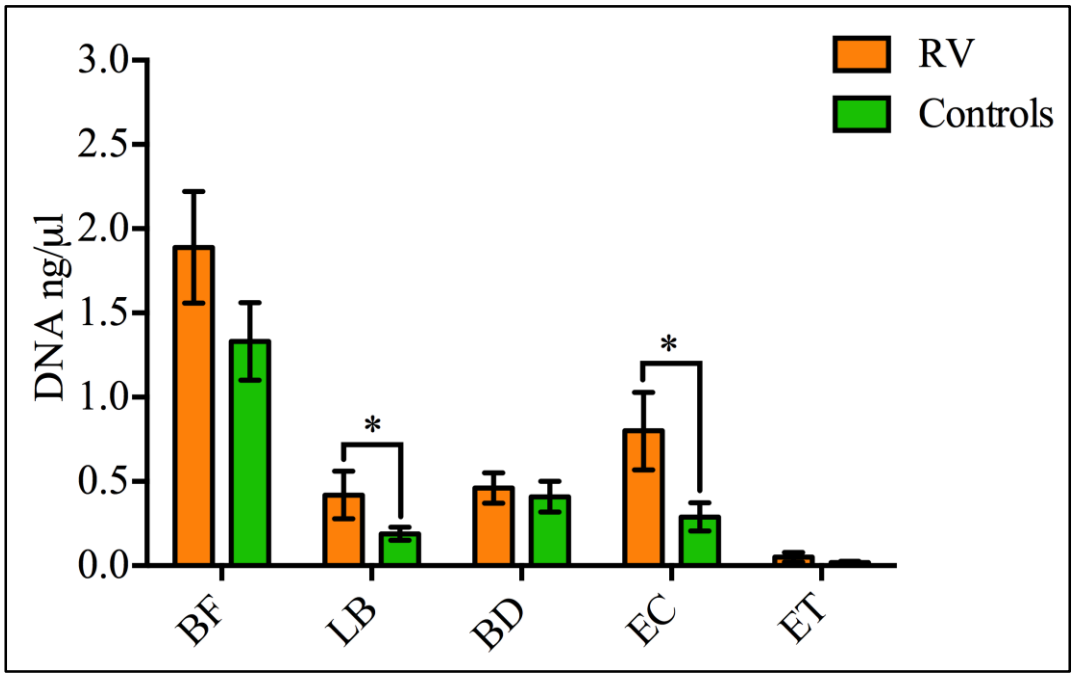

Figure 3.2. Bacterial DNA levels in rotavirus infection. Mucosally protective *Lactobacillus* spp. levels were significantly elevated in RV infection compared to levels in healthy controls. Potentially pathogenic *E. coli* levels were also significantly elevated compared to levels in healthy controls. The mucosally protective *Bifidobacterium* spp. levels were higher than *E. coli* levels in RV infection. *Enterococcus* spp. and *Bacteroides* spp. levels were not significantly altered compared to controls in RV infection. RV, rotavirus; BF, *Bifidobacterium* spp.; LB, *Lactobacillus* spp.; BD, *Bacteroides* spp.; EC, *E. coli* and ET, *Enterococcus* spp. *p<0.05, data shown as ± SEM.

3.6.2 Microbiota changes in human boca virus 2 infection

In HBoV2 infection no correlation with faecal levels of any microorganisms tested and bowel frequency was observed (Figure 3.3). Interestingly, the frequency of bowel movements showed a wider range compared to observations in RV infection (Table 3.3). However, there was no correlation between frequency of bowel movements and *Bifidobacterium* spp. in HBoV2 infection (Figure 3.3). Endogenous levels of beneficial *Lactobacillus* spp. were significantly elevated (0.31 ± 0.05 ng/µL) in HBoV2 infection compared to healthy controls (0.20 ± 0.04 ng/µL, p=0.03) (Figure 3.4). The opportunistic pathogens, *Enterococcus* spp. (0.03 ± 0.005 ng/µL) and *E. coli* levels (1.63 ± 0.63 ng/µL) were significantly elevated compared to healthy controls (0.02 ± 0.007 ng/µL and 0.30 ± 0.08 ng/µL, p=0.006 and p=0.004, respectively) (Figure 3.4). *Bifidobacterium* spp. and *Bacteroides* spp. levels were similar to those seen in the healthy control group (Figure 3.4).

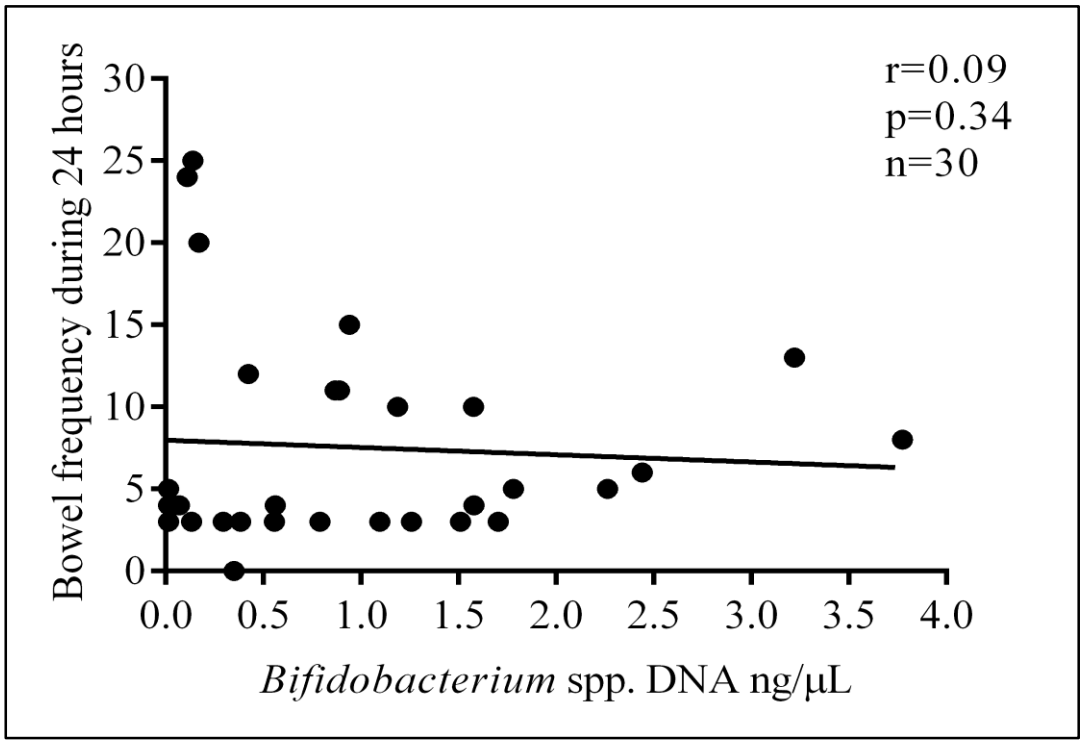

Figure 3.3. *Bifidobacterium* spp. DNA levels compared with frequency of bowel movements in human boca virus 2 infection. Decreased bowel movements were not correlated with *Bifidobacterium* spp. levels in HBoV2 infection over a 24 h period.

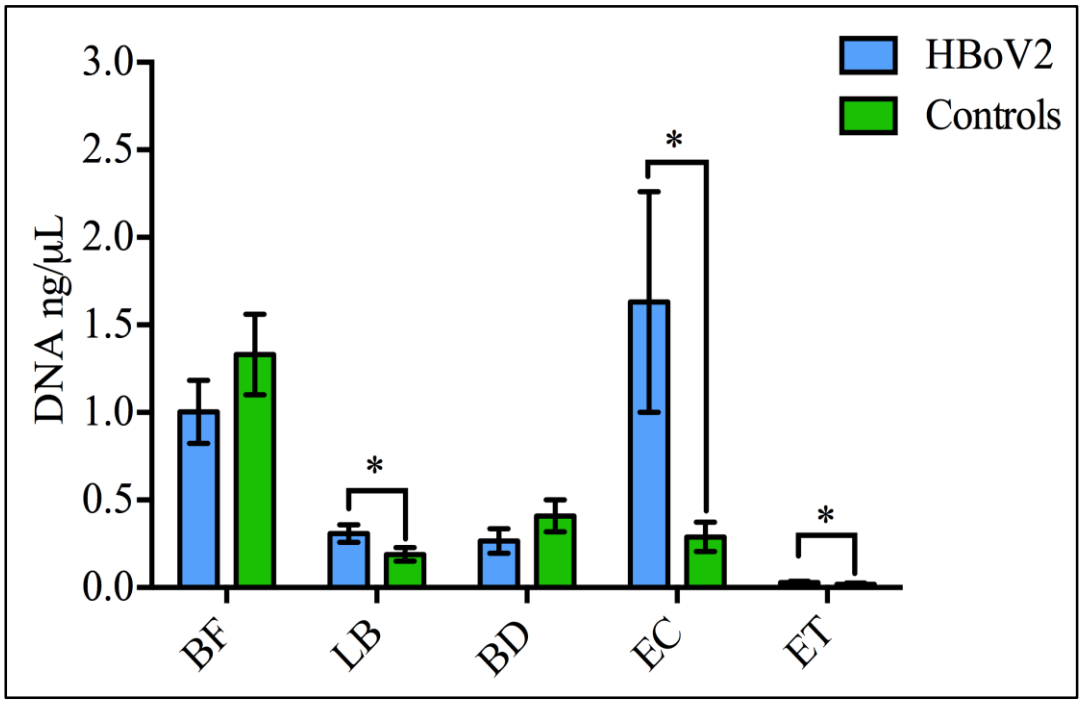

Figure 3.4. Bacteria DNA levels during human boca virus 2 infection. HBoV2 infection compared to bacterial levels in healthy controls. *E. coli* levels were significantly raised in HBoV2 infection compared with levels observed in healthy controls. *Lactobacillus* spp. levels were also significantly elevated in HBoV2 infection compared with healthy controls. *Enterococcus* spp. levels were higher in the HBoV2 group when compared to healthy controls. *Bifidobacterium* spp. and *Bacteroides* spp. levels were not significantly different compared to controls in HBoV2 infection. HBoV2, human boca virus 2; BF, *Bifidobacterium* spp.; LB, *Lactobacillus* spp.; BD, *Bacteroides* spp.; EC, *E. coli* and ET, *Enterococcus* spp. *p<0.05, data shown as ± SEM.

3.6.3 Microbiota changes in rotavirus and human boca virus 2 infections

Endogenous *Bifidobacterium* spp. levels were significantly higher in RV infection (1.90 ± 0.3 ng/µL) compared to levels in HBoV2 infection (1.00 ± 0.18 ng/µL) (p=0.04) (Figure 3.5). Interestingly, *Bifidobacterium* spp. levels (1.90 ± 0.30 ng/µL) were raised compared to *E. coli* levels (0.80 ± 0.23 ng/µL) in RV infection (Figure 3.5). *E. coli* levels were elevated (1.63 ± 0.63) compared with *Bifidobacterium* spp. levels (1.00 ± 0.18) in HBoV2 infection (Figure 3.5).

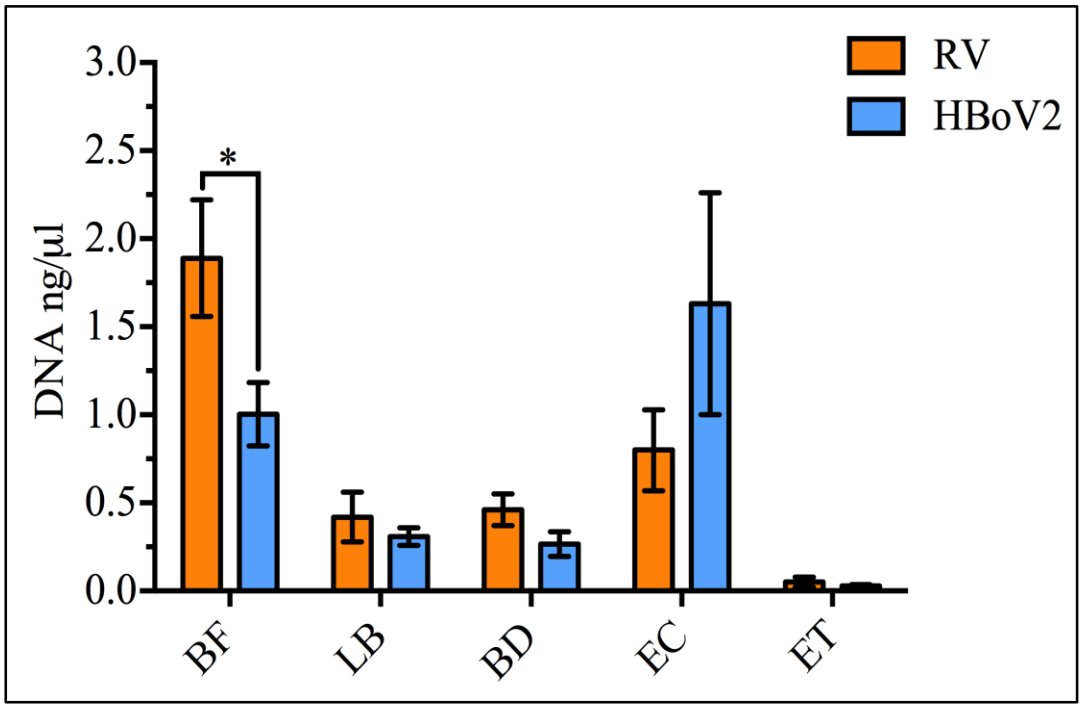

Figure 3.5. Bacterial DNA levels in rotavirus and human boca virus 2 infection. *Bifidobacterium* spp. levels were significantly raised in RV infection compared to levels in HBoV2 infection. *E. coli* levels were decreased compared to *Bifidobacterium* spp. levels in RV infection. In contrast *E. coli* levels were elevated compared with *Bifidobacterium* spp. levels in HBoV2 infection. *Lactobacillus* and *Bacteroides* spp. levels were not significantly different in Both RV and HBoV2 infection. RV, rotavirus; HBoV2, human boca virus 2; BF, *Bifidobacterium* spp.; LB, *Lactobacillus* spp.; BD, *Bacteroides* spp.; EC, *E. coli* and ET, *Enterococcus* spp. *p<0.05, data shown as ± SEM

3.7 Discussion

One important finding of this study was demonstrated in the RV group where higher levels of beneficial endogenous *Bifidobacterium* spp. correlated with fewer bowel motions in a 24 h period. However, in the HBoV2 group no such associations were seen. The data suggests a differential adaptive response in the faecal microbiome in patients with RV and HBoV2 infection compared with healthy age matched controls. This may be due to a different mechanism of action between RV vs. HBoV2 infections.

The microbiome represents a diverse array of microbes playing an essential role in human health influencing immunity, protection from pathogens, nutrient assimilation, physiology, host gene expression and behaviour (Dethlefsen, Mcfall-Ngai and Relman 2007, Forsyth, Shannon, Kordower *et al.* 2011, Stearns, Lynch, Senadheera *et al.* 2011). Viral infections of the GIT can disrupt the functioning of the GIT manifesting as acute severe diarrhoea. Disruptions to gastrointestinal ecology thought to occur during viral gastroenteritis can if they persist lead to long-term alterations in the microbial composition causing mucosal stress and immune system dysregulation (Thanabalasuriar and Kubes 2014). However, once the infection has resolved changes to the luminal and mucosal environments may potentially induce various GIT disorders as has been shown with post infectious irritable bowel syndrome (PI-IBS) (Spiller and Campbell 2006). The significance of the changing microbial environment during RV and HBoV2 infection and the long term developmental effects on the microbiome during maturational development of infancy, and its relatedness to disease susceptibility later in life is unclear. It must be emphasised

that the current study was in children from the developed world, with post-infectious sequelae more likely to be more evident in infants of the developing world.

Changes to the microbiome following antibiotic administration in neonatal mice have shown altered granulocyte regulation compared to mice with a diverse microbiome (Deshmukh, Liu, Menkiti *et al.* 2014). Specifically, bacterial products were shown to stimulate granulopoiesis, via toll like receptor 4 (TLR4) and interleukin–17 (IL-17) production, with circulating neutrophils playing a role in sepsis resistance in neonates (Deshmukh, Liu, Menkiti *et al.* 2014). Another study has shown that young mice are at increased risk of developing allergic asthma when the microbiota is altered compared to mice with a healthy unaltered microbiota (Russell, Gold, Hartmann *et al*. 2012). These findings in mice also coincide with findings in human studies with infants receiving antibiotics. Studies have also shown a relationship between antibiotic exposure early in life and the incidence of atopy and asthma (Marra, Marra, Richardson *et al*. 2009, Schmitt, Schmitt, Kirch *et al*. 2010). Other studies investigating the incidence of allergy and asthma during childhood have shown altered populations of bifidobacteria with differing *Bifidobacterium* spp. associated with allergy (Štšepetova, Sepp, Julge *et al*. 2007). These studies indicate the importance of *Bifidobacterium* spp. in the early phase of development of the paediatric microbiome. The present study represents an acute disease, and shows alterations in the intestinal microbiome compared to healthy controls with known morphological changes in the small intestinal mucosa. In both animal and human studies alterations in the microbiome have resulted in increased incidence of allergic diseases. This study along with others highlight the need for both short and longer term monitoring

of the faecal microbiome with particular focus on beneficial bacteria during childhood and burden of disease. This may be particularly pertinent in the setting of persistent or chronic diarrhoea.

Many studies investigating probiotics for the treatment of RV infection and diarrhoea have focused on exogenous probiotic *Lactobacillus* GG, which has shown efficacy in reducing both severity and duration of diarrhoea (Vanderhoof, Whitney, Antonson *et al.* 1999, Guandalini, Pensabene, Zikri *et al.* 2000, Rosenfeldt, Michaelsen, Jakobsen *et al*. 2002, Van Niel, Feudtner, Garrison *et al*. 2002, Szajewska, Skorka, Ruszczyński *et al.* 2013). This is in contrast to a study investigating diarrhoeal disease in Australian aboriginal children and the use of *Lactobacillus* GG in the treatment thereof. This study failed to find any beneficial effects of *Lactobacillus* GG in the ability to decrease episodes and duration of diarrhoea (Ritchie, Brewster, Tran *et al*. 2010). This may in part be due to the known environmental enteropathy present in indigenous children in the top end of Australia and perhaps exacerbated by lower levels of endogenously present beneficial *Lactobacillus* spp. and *Bifidobacterium* spp. as potential modifiers of the severity of diarrhoea in these children. The efficacy of *Lactobacillus* GG to decrease duration and severity of diarrhoeal illness during RV infection may owe its efficacy in part to the existing levels of both beneficial endogenous *Lactobacillus* and *Bifidobacterium* spp. prior to and during RV infection.

A study using Balb/c mice infected with rhesus RV and treated with bifidobacteria alone and with a prebiotic showed that bifidobacteria alone decreased diarrhoeal symptoms in

the treated mice compared with controls (Qiao, Duffy, Griffiths *et al.* 2002). They reported that RV specific immunoglobulin A was increased 4 fold in the RV infected group treated with bifidobacteria compared with the RV infected group without bifidobacteria (Gigante, Tortora, Ianiro *et al*.). RV specific IgA has been shown to neutralize both cell bound and unbound RV (Velazquez, Matson, Guerrero et al. 2000). *Bifidobacterium* and *Lactobacillus* spp. have been shown to increase specific RV IgA levels in human studies (Kaila, Isolauri, Saxelin *et al*. 1995). In the present study increased levels of *Bifidobacterium* spp. were associated with decreased bowel movements. A possible explanation for this may be due to increased circulating levels of the specific RV IgA stimulated by the endogenous *Bifidobacterium* spp. leading to decreased mucosal stress and therefore diarrhoeal symptoms. Although this current study did not test circulating IgA levels, previous studies have shown increased levels of IgA are associated with bifidobacteria (Velazquez, Matson, Guerrero *et al.* 2000). Others have also shown that *Bifidobacterium longum* has a potentially protective effect via the production of acetate in preventing enteropathogenic *E. coli* infection. Using a mouse model of enteropathogenic *E. coli* infection, Fukunda *et al* 2011, reported that certain strains of Bifidobacteria (*B. longum*) prevented *E. coli* infection (Fukuda, Toh, Hase *et al.* 2011). These studies indicate that *Bifidobacterium* spp. are important commensal bacterium colonizing the intestinal tract of infants and altered levels may be deleterious. Another study has shown a specific adhesion protein secreted by *Bifidobacterium adolescentis* mediated the release of pro-inflammatory cytokines and promoted mucosal repair (Shu, Yu, Kang *et al.* 2014). These examples demonstrate that specific species of bifidobacteria have a protective role in decreasing the effects of mucosal stress. These studies combined with

the current study findings highlight the importance of understanding the endogenous bacterial species present at time of infection and their role in pathogenesis of disease.

The GIT of infants is colonized early with *Lactobacillus* and *Bifidobacterium* spp. These endogenous bacteria are important in the protection of the mucosa, mediating intestinal permeability and priming the immune system. Germ free animals with no microbial involvement manifest a heightened stress response, which is corrected when colonization occurs with a diverse array of microorganisms or monoassociation with *Bifidobacterium infantis* (Sudo, Chida, Aiba *et al*. 2004). There appears to be a critical window during early development in which many systems are deleteriously affected by an altered microbiome. Indeed, studies show the relationship between the microbial colonisation of the GIT and the mucosa is required for the correct functioning of the hypothalamic-pituitary-adrenal axis and the response to stress later in life (Shanahan 2012). This current study has shown that both *Lactobacillus* and *Bifidobacterium* spp. are differentially altered in two different viral infections affecting mucosal protection and therefore mucosal stress, with the long-term implications poorly understood.

In our current study endogenous *Bifidobacterium* spp. concentrations were significantly higher than *E. coli* levels in RV infection. However, in HBoV2 infection *Bifidobacterium* spp. were significantly lower than *E. coli* levels observed in RV infection. *Bifidobacterium* spp. were lower compared to *E. coli* levels in HBoV2 infection, but not significantly. Interestingly, *E. coli* was raised in HBoV2 infection compared with RV infection. These results indicate that the microbiota responds differently in RV infection compared to

HBoV2 infection, and that the raised levels of *Bifidobacterium* spp. observed in RV infection may mediate the overall toxic load from RV and the potential pathogen *E. coli*. Ma *et al* 2011, reported marginally raised levels of *Bifidobacterium* spp. compared to control levels in RV infection in a Chinese paediatric population with different gastrointestinal viral illnesses (RV, astrovirus, norovirus and adenovirus) (Ma, Wu, Nawaz *et al.* 2011). Endogenous *Lactobacillus* spp. concentrations were significantly raised in faeces compared to control levels in RV infection. Decreased levels of *Lactobacillus* spp. were also reported in RV infection. In contrast, our study observed significantly higher levels of *Lactobacillus* spp. in RV and HBoV2 infections compared to controls. Reasons for this difference could include geographical, socioeconomic and dietary influences. The interactions of commensal bacterial pathogens and potentially protective endogenous microbes, deserves further study in the context of the severity and persistence of symptoms.

Different levels of endogenous bacterial species observed in this study may be due to variance of ecological niches for both viral pathogens. The differences observed in levels of *Bifidobacterium* and *Lactobacillus* spp. in both viral infections show altered adaptive patterns of the microbiome related to the region of the GIT infected. Indeed, RV is known to target the mature enterocytes in the jejunum and ileum (Colbere-Garapin, Martin-Latil, Blondel *et al.* 2007) causing villous necrosis, resulting in impaired electrolyte regulation leading to profuse watery diarrhoea (Ramig 2004). The RV toxin, NSP 4 is believed not to affect the epithelial layer of the large intestine. The pathogenesis of HBoV2 is not known and may affect a different gastrointestinal region and have a different mechanism

of action. The beneficial endogenous species quantified in this study have different ecological niches and functions within the GIT. *Lactobacillus* spp. are ecologically niched to the microaerophilic region of the small intestine, whereas *Bifidobacterium* spp. are niched to the anaerobic environment of the large intestine. It is possible that decreased levels of *Bifidobacterium* spp. observed in HBoV2 infection may indicate HBoV2 disrupts the ecological equilibrium within the large intestine directly decreasing *Bifidobacterium* spp. Raised levels of the commensal and potentially pathogenic bacteria, and the balance of these at the time of viral infection may account for differing symptom responses and an altered time course resolution after viral infection.

There is a growing body of evidence suggesting the microbiome plays a significant role in host susceptibility to pathogens (Pfeiffer and Sonnenburg 2011). The data in the current study suggests that both viruses alter the composition of the faecal microbiome, leading to increases in the potentially pathogenic bacteria *E. coli* and *Enterococcus* spp. compared with healthy controls, with concomitant alterations in the endogenous beneficial populations.

3.8 Conclusion

It is increasingly recognized that the faecal microbiome is fundamental for human health and alterations to the microbiome may detrimentally affect host function both acutely and in the long term. This study has shown that the faecal microbiome adapts differentially in two separate viral infections. In the case of RV infection the higher levels of *Bifidobacterium* spp. were associated with improved diarrhoeal symptoms. No such relationship was seen in HBoV2 infected children. In contrast, the levels of *E. coli* were elevated in both viral infections compared to controls. The *Bifidobacterium* spp. levels in HBoV2 infection were significantly lower than those observed in RV infection. It could be hypothesised that the combination of these two factors i.e. a higher putative pathogen (*E. coli*) load and a lower beneficial microbe measurement played a role in the symptomatic response. Further analysis of ratios of putative pathogens to beneficial microbes deserves attention. Moreover, the host's susceptibility to viral infections may be dependent on the prevailing bacterial community present at the time of infection. Implications of these preliminary observations for ecological modification of the microbiome in the prevention and targeted treatment of childhood diarrhoea are promising. Larger studies both retrospective and prospective are now required to confirm this data. This would likely lead to design of personalised interventions investigating the impact of exogenous probiotics based on those detected endogenously.

Chapter 4.0 The intestinal microbiome and non-invasive parameters of gastrointestinal mucosal stress in a cohort of IBS patients

4.1 Preface

IBS is a widely occurring functional gastrointestinal illness with no clear aetiological precursors, and no recognized biomarkers to aid with diagnosis. IBS diagnosis is further complicated by the symptomatology and classification of specific subtypes. Chapter 4 explores the relationship between the bacterial composition of the microbiome and non-invasive markers of mucosal stress in a well-classified population of IBS patients, with the aim of identifying possible indicators of mucosal stress. This study has shown that certain endogenous bacteria of the microbiome corresponded with non-invasive markers of mucosal stress, suggesting that these microorganisms may have a potential role in decreasing small intestinal permeability and modulating intestinal inflammation. The microbiome represents a vast resource of potential therapeutic targets for the possible treatment and management of IBS. Moreover, this study shows certain endogenous bacteria may have a role as indicators of mucosal dysfunction in certain subsets of IBS patients.

4.2 Abstract

Introduction

Irritable bowel syndrome (IBS) is an idiopathic illness with evidence suggesting microbial involvement as either an initiator, promoter or potentially protective. Intestinal permeability is emerging as a contributor to intra- and extra-intestinal disease. The aim of this study was to investigate the relationship between the microbiome and non-invasive markers of mucosal integrity in IBS patients.

Materials and Methods

Faecal DNA from adult IBS patients (n=34) was analysed using real-time PCR with previously optimized primer sets targeting 16s rRNA. Lactulose/Rhamnose ratio in blood samples was used to assess mucosal permeability. Faecal calprotectin was used to measure intestinal inflammation.

Results

Higher endogenous *Lactobacillus* spp. and *Bifidobacterium* spp. levels ($r=-0.52$, $p<0.003$, and $r=-0.59$, $p<0.001$, respectively) were associated with improved small intestinal permeability determined by L/R ratio. Faecal concentrations of *F. prausnitzii* and *Rosburia* spp. were also significantly correlated with L/R ratio, indicative of a more patent intestinal mucosa ($r=-0.48$, $p<0.01$ and $r=-0.60$, $p<0.01$, respectively). Only *Bifidobacteria* spp. ($r=-0.38$, $p<0.03$) and *F. prausnitzii* ($r=-0.42$, $p<0.02$) showed a relationship to levels of faecal calprotectin at recruitment. This study has also shown bacterial changes between the different categories of IBS and non-invasive markers of mucosal stress. Selected bacteria of the intestinal microbiome correlated with reduced intestinal permeability in IBS and in different IBS classifications.

Conclusions

The probiotic *Bifidobacterium* spp. and the reportedly anti-inflammatory bacteria *F. prausnitzii* may have a role in decreasing small intestinal permeability and modulating inflammatory pathways in the GIT. The data suggests increasing levels of known endogenous probiotic bacteria may tighten barrier function, while other putatively known probiotic bacteria may have a restorative active on traumatized mucosa.

4.3 Introduction

The human GIT harbours trillions of microorganisms (the microbiome) that have a direct effect on host response and functioning of mucosae lining the GIT (Hooper and Gordon 2001, Chow, Lee, Shen *et al.* 2010). The barrier lining the GIT is fundamental in maintaining the divide between the intestinal microbiome and the internal milieu (Davidson, Kritas and Butler 2007). Moreover, the mucosal barrier mediates the crosstalk between the two, maintaining gastrointestinal homeostasis. Any insult that stresses the compositional balance of the microbiome can alter barrier function promoting mucosal dysfunction and results in increased intestinal permeability and inflammation (stressed mucosa) (Davidson, Kritas and Butler 2007).

IBS is a syndrome of unknown aetiology, and characterised as one where barrier abnormalities and compositional changes in the microbiome are potential drivers of symptoms (Codling, O'mahony, Shanahan *et al.* 2010). IBS is classified according to the dominant symptoms, including diarrhoea dominant (IBD-D), constipation dominant (IBS-C), alternating constipation and diarrhoea (IBS-A), and suspected mode of acquisition such as post infectious IBS (IBS-PI). Probiotics and certain endogenous bacterial species have been reported to alter intestinal permeability and decrease inflammation in IBS patients (Sokol, Seksik, Furet *et al.* 2009, Miquel, Martin, Rossi *et al.* 2013, Ford, Quigley, Lacy *et al.* 2014, Wang, Gong, Wang *et al.* 2014). The probiotic bacteria *B. lactis* has shown the capacity to decrease pain, abdominal distension, and increase transit time in IBS-C patients (Agrawal, Houghton, Morris *et al.* 2009). Decreased levels of the endogenous and mucosally protective *F. prausnitzii* have been reported in patients with Crohn's disease and IBS (Sokol, Seksik, Furet *et al.* 2009, Rajilić–Stojanović, Biagi, Heilig

et al. 2011, Miquel, Martin, Rossi *et al.* 2013). Further to this, the difference in the levels of *F. prausnitzii* could help in the differential diagnosis between IBD and IBS patients (Lopez-Siles, Martinez-Medina, Busquets *et al.* 2014).

Increased intestinal permeability and compositional changes in the microbiome have been reported in IBS patients with diarrhoea as a dominant symptom (Dunlop, Hebden, Campbell *et al.* 2006, Lyra, Rinttilä, Nikkilä *et al.* 2009, Mujagic, Ludidi, Keszthelyi *et al.* 2014, Shukla, Ghoshal, Dhole *et al.* 2015). Furthermore, compositional shifts in the microbiome have been reported in other IBS phenotypes such as IBS-C and A compared to healthy controls (Lyra, Rinttilä, Nikkilä *et al.* 2009, Shukla, Ghoshal, Dhole *et al.* 2015). Intestinal permeability was more severe in non-post infectious IBS patients when compared with post-infectious IBS patients and controls. Furthermore, IBS patients with eczema, asthma or hayfever had greater small intestinal permeability than those IBS patients without atopy, suggesting that increased intestinal permeability may be associated with these pathologies (Dunlop, Hebden, Campbell *et al.* 2006). Lyra *et al* 2009 has shown compositional changes in specific bacterial populations across IBS-phenotypes suggesting that specific bacterial communities may also play a role in each phenotype (Lyra, Rinttilä, Nikkilä *et al.* 2009).

Faecal microbiomes were also altered in IBS-D patients compared to IBS-C patients when compared to controls. In addition, IBS-D and IBS-C patients had higher number of *Pseudomonas aeruginosa* compared to controls. These studies suggest that compositional changes in the microbiome and the predominance of mucosal changes in certain subtypes of IBS may be a driver of intestinal symptoms. Alterations in luminal

bacteria may favour opportunistic pathogens like *P*. *aeruginosa* (Shukla, Ghoshal, Dhole *et al*. 2015). Furthermore, *P*. *aeruginosa* excrete virulence factors such as proteases that alter intestinal permeability and may affect gut motility (Kerckhoffs, Ben-Amor, Samsom *et al.* 2011). In other studies, increased levels of beneficial *Lactobacillus* spp. have been reported in IBS-D patients compared to healthy controls and decreased levels of *Bifidobacterium catenulatum* and *Clostridium coccoides* subgroup compared with controls (Malinen, Rinttilä, Kajander *et al.* 2005). Compositional changes in the microbiome appear to be associated with different subtypes of IBS. These compositional changes may be associated with specific syndrome symptoms, which promote gastrointestinal dysfunction.

The focus of this retrospective analysis was to determine whether mucosally protective microbes associated with gut and mucosal health were associated with markers indicative of mucosal stress. This was achieved by quantitative analysis of the faecal microbiome in a cohort of IBS patients fulfilling the ROME 11 criteria. This information may aid in the development of new diagnostic approaches aimed at better understanding the role of the microbiome and its relationship with stressed mucosa.

4.4 Material and Methods

The analyses in this study were carried out on baseline (pre-randomisation) data collected from the participants of a randomised blinded control trial (RCT), designed to investigate the potential benefits of Lyprinol (a green-lipped mussel extract) in the treatment of the low grade inflammation in IBS. This study was carried out at the Women's and Children's Hospital, Adelaide, South Australia in the Department of Gastroenterology. Ethical approval for this study was obtained through the Ethics Committee of the Women's and Children's Hospital (Approval No. 1855). One stool sample was taken at the beginning of the study at enrolment and analysed using PCR. Blood samples were collected at enrolment and stored at -80°C until analysed. Breath testing for small bowel bacterial overgrowth (SBBO) was carried out using a combination dual sugar test for small intestinal permeability and the H_2 breath test (Ritchie, Brewster, Davidson *et al.* 2009).

4.4.1 Participants

In this study 34 IBS patients were recruited. Of these 20 were categorized as IBS-D, 8 as IBS-C and 6 as IBS-A (Table 4.1). All patients met the ROME 11 diagnostic criteria and were recruited from either an established database and through promotional activity (television and flyers in the community) and subsequent contact with the Gastroenterology department at the Women's and Children's Hospital. Patients were excluded from the study if they had seafood allergy, any type of malignancy, gastrointestinal surgery, chronic pre-existing disease (cardiovascular, gastrointestinal, immunological, etc.), and used any non-steroidal anti-inflammatory drugs.

Table 4-1: Characteristics and measurements in IBS patients at recruitment. FC, faecal calprotectin; L/R, lactulose/rhamnose ratio; SBBO, small bowel bacterial overgrowth; IBS-D, diarrhoea predominant irritable intestine syndrome. Values are shown as mean ± SD

Patient characteristics	Patient data
Male:Female	9:25
Age in yrs. (mean ± SD)	49.8 ± 17.0
IBS	34
L/R ratio (x100) (mean ± SD)	(17.18 ± 11.93)
FC (µg/g) (mean ± SD)	(63.93 ± 52.73)
SBBO (positive H_2 breath test)	3 (1M:2F)
Faeces for RT-PCR	34

4.4.2 Small intestinal permeability testing

Intestinal permeability was measured using the Lactulose/Rhamnose (L/R) ratio in blood. Patients were fasted overnight prior to receiving lactulose/rhamnose solution, with physical activity kept to a minimum. A solution of 7.5 mL lactulose (67% w/v) syrup (Duphalac; Solvay Pharmaceuticals, Sydney, NSW) and 1.1 g α-L-rhamnose (Merck Pty Ltd, Kilsyth, Victoria, Australia) made up to a total volume of 100 mL with water and adjusted to an osmolality of 400 mOsm/L was ingested. Blood was taken by venepuncture (10 mL) at 90 minutes post-ingestion of the oral sugars, with plasma analysed for concentration of lactulose and rhamnose using high performance liquid chromatography (HPLC) as previously described (Haase, Kukuruzovic, Dunn *et al.* 2000). A normal L/R ratio from our laboratory is <8.8.

4.4.3 Faecal calprotectin

Faecal calprotectin concentrations were assessed with a Quantitative Lateral Flow Assay (Bühlmann Laboratories AG, Switzerland). The test is designed for the selective measurement of faecal calprotectin antigen by sandwich immunoassay. Samples were diluted using the extraction buffer provided and loaded onto the test cartridge for analysis in the BÜHLMANN Quantum Blue® Reader. The published standard level for a normal faecal calprotectin is <50 µg/g; (Bühlmann Laboratories AG, Switzerland).

4.4.4 Breath testing

Testing for SBBO was undertaken using H_2 breath test after ingestion of lactulose. Study participants were required to fast overnight (no solids or liquids for at least 10 h), prior to the test (small amounts of water were permitted during this time). Participants were

instructed to eat a low fibre meal the day before testing and not to exercise on the day of the test. A baseline breath test was taken, and then a drink was ingested containing 5 g lactulose (Genepharm Pty Ltd, Victoria, Australia) and 1 g rhamnose (Sigma) in 100 mL of water. Samples of breath were collected every 15 min for 90 min, then every 30 min until 180 min. Samples were analysed using BreathTracker SC (Quintron, Milwaukee, USA). A positive breath test is an elevated H_2 reading of >10ppm above baseline during the initial 60 min of testing, and is an indicator of SBBO.

4.4.5 DNA extraction

DNA was extracted from 0.25 g of faeces using the bead beating plus column method described by Morrison and Yu (Yu and Morrison 2004). Briefly, faeces were thawed on ice, then homogenised with garnet beads (0.70 mm) in lysis buffer. Lysis buffer consisted of 29.22 g NaCl, (S-3014, Sigma), 6.075 g Tris base (T6066, Sigma) and 18.612 g Ethylenediaminetetraacetic acid (EDTA, E5134, Sigma) in 800 mL milliQ water, pH 8. 40 g Sodium dodecyl sulfate (SDS, L4390, Sigma) was then added and dissolved, pH adjusted to 8, and made up to 1L using milliQ water. After homogenisation, samples were incubated at 70°C for 15 minutes. Precipitation of nucleic acid was achieved with a set of incubation and centrifugation steps using ammonium acetate, isopropanol and 70% ethanol. Nucleic acid pellet was dissolved in sterile Tris-EDTA (TE) buffer (12.1 g/L Tris base, 3.72 g/L EDTA in milliQ water, pH 8). Total DNA concentration and purity (260 nm:280 nm ratio) were determined using a Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, Scoresby, Australia). DNA was diluted to a working concentration of 5 ng/µL.

4.4.6 Quantitative real-time PCR

All quantitative PCR (qPCR) analyses were performed on a CFX 384TM real-time PCR detection system (Bio-Rad, Hercules, CA) in triplicate in a total volume of 10 µL for each reaction. Each reaction consisted of 3 µL (1 ng/µL–3 ng/µL) DNA template and 6 µL PCR mixture containing 5 µL SsoFast EvaGreen Supermix, 0.5 µL bovine serum albumin, forward and reverse primers and PCR-grade water. Primers used in this study have been previously used (Table 4.2). The PCR cycling conditions were as follows: hotstart at 98°C for 3 min followed by 35 cycles of 2-step PCR with denaturing at 98°C for 15 s and annealing/elongation time and temperature as given below. Melt curve analyses were performed after completion of all cycles, with fluorescence acquired at 0.5°C intervals between 55°C and 95°C to verify amplification specificity. A series of eight 10-fold dilutions containing the target amplicon were analysed in parallel with DNA samples for estimation of absolute abundance and PCR efficiency. Data were analysed with Bio-Rad CFX Manager software (version 1.5) for absolute quantities. Bacterial levels are reported in ng/µL. Data were normalized by log10 transformation.

Table 4-2: Cycling conditions of primers used in this study.

Primer	Reactive organisms	Anneal temp and time	Extension	Cycles	Product size
Bifidobacterium spp. (Rinttilä, Kassinen, Malinen *et al.* 2004)	*B.breve, B.bifidum, B. adolescentis B. longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminatum, B. thermophilium, B. subtile, B. bifidum, B. boum, B. animalis, B. choerium, B. gallicum, B. pseudolongum, B. magnum, B. infantis, B. indicum, B. gullinarum, B. pullorum, B. saeculare, B. suis.*	60°C, 50s	30s	45	243
Lactobacillus spp. (Rinttilä, Kassinen, Malinen *et al.* 2004)	*L.acidophilus, L. amylovorus, L. delbrueckii, L. amylolyticus, L. acetotolerans, L. crispatus, L. amylophilus, L. johnsonii, L. gasseri, L. fermentum, L. pontis, L. reuteri, L. mucosae, L. vaginalis, L. panis, L. oris, L. pentosus, L. plantarum, , L. fructivorans, L. mali, L. animalis, L. murinus, L. ruminis, L. agilis, L. salivarius, L. aviarius, L. sharpeae, L. manihotivorans, L. rhamnosus, L. casei, L. zeae, L. sakei, Pediococcus pentosaceus, P. parvulus, P. acidilactici, P. dextrinicus, Weissella halotolerans, W. confusus, W. Paramesenteroides, W. hellenica, W. viridescens, W. kandleri, W. minor, Leuconostoc Lactis.*	50°C, 20s	30s	45	341
E. coli (Rinttilä, Kassinen, Malinen *et al.* 2004)	*E. coli.*	60°C, 20s	30s	35	95
F. prausnitzii (West, Christophersen, Pyne *et al*. 2013)	*F. prausnitzii.*	60°C, 20s	30s	35	198
Rosburia spp.	*Rosburia spp.*	58°C, 45s	30s	35	230
R. torques	*R. torques*	58°C, 40s	40s	35	151
R. gnavus	*R. gnavus*	58°C, 20s	30s	35	130
R. bromi	*R. bromi*	60°C, 30s	30s	35	183
A. muciniphila (Halmos, Christophersen, Bird *et al.* 2014)	*A. muciniphila*	63°C, 30s	30s	35	327

4.4.7 Statistical analysis

Statistical analysis of data from the quantitation of bacterial levels in faecal samples using PCR was carried out using Graphpad Prism 6 software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). Pearson's correlation and Spearman's correlation were used to correlate changes between intestinal parameters of mucosal stress and faecal levels of selected bacteria. Normality of data was tested using D'Agostino & Pearson omnibus normality test. Non-parametric data was analysed using the Mann Whitney U test, with Bonferroni test used to determine significance of p values. $p < 0.05$ was considered as significant. Data are expressed as mean ± SEM.

4.5 Results

The data presented here is of well-categorized IBS patients. The data is presented in the general IBS cohort irrespective of classification, and then separated into the three dominant categories IBS-D, IBS-C and IBS-A. There were significant correlations observed between non-invasive markers of mucosal stress and selected endogenous bacteria of the microbiome in the general population and the specific categories of IBS.

4.5.1 Faecal calprotectin levels in IBS patients at recruitment

One patient in this cohort had an extremely elevated faecal calprotectin reading (385.6 µg/g) at recruitment and was excluded from analysis. Mean levels of faecal calprotectin at recruitment are shown in table 4.1. Faecal calprotectin levels ranged from 6 µg/g to 182.5 µg/g, with 14 patients > 50 µg/g, and 16 patients < 50 µg/g (Table 4.3), Levels of *Bifidobacterium* spp. were significantly correlated with intestinal inflammation as measured by faecal calprotectin (r=-0.38 (95% CI (95% CI -0.7948 to -0.1037), p=0.03) (Figure 4.1). In the general IBS cohort faecal levels of *F. prausnitzii* were significantly correlated with faecal calprotectin levels at recruitment (r=-0.48 (95% CI-0.7141 to -0.1532), p=0.006) (Figure 4.1). Levels of faecal calprotectin were not significantly different between the different IBS classifications (Figure 4.2). There was also some significant associations observed between selected endogenous bacteria in the faecal microbiome in the general IBS cohort (Appendix 4.1)

Table 4-3: Range of Faecal calprotectin levels in all IBS patients at recruitment.
FC, faecal calprotectin in µg/g, Levels are shown as mean ± SEM

	Number of IBS patients	FC, µg/g
FC > 50 µg/g	n=14	108.40 ± 43.60
FC < 50 µg/g	n=16	22.98 ± 13.11

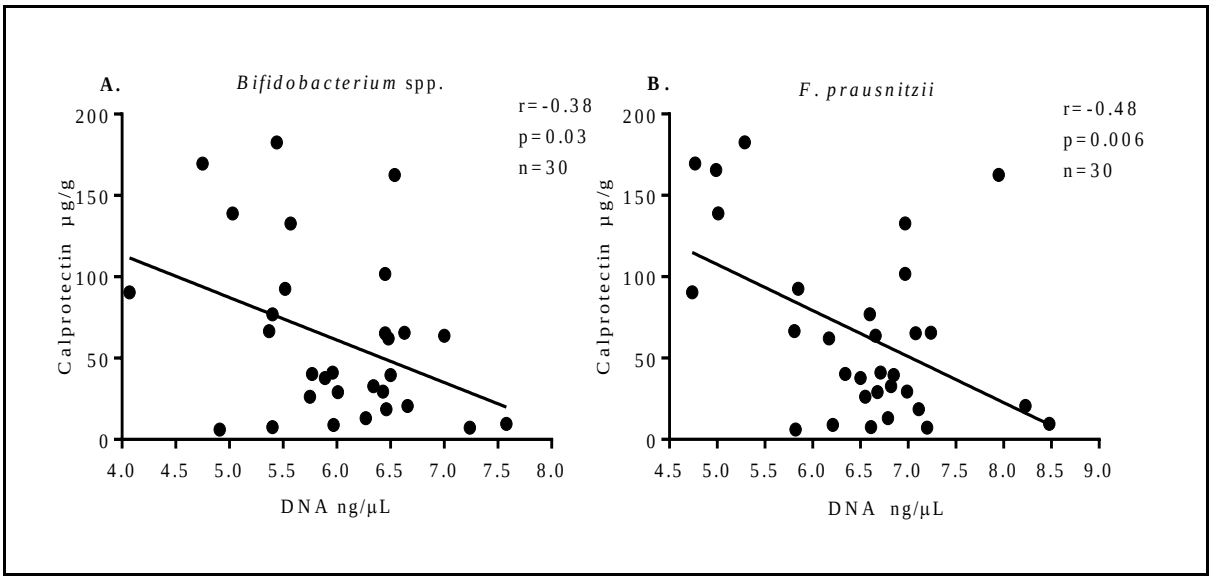

Figure 4.1. Levels of endogenous beneficial bacteria and faecal calprotectin in IBS patients at recruitment. (**A**) Levels of the mucosally protective *Bifidobacterium* spp. were correlated with intestinal inflammation (r=-0.38 (95% CI (95% CI -0.7948 to -0.1037), p=0.03), (**B**) Faecal calprotectin levels were also correlated with faecal levels *F. prausnitzii* (r=-0.48 (95% CI-0.7141 to -0.1532), p=0.006).

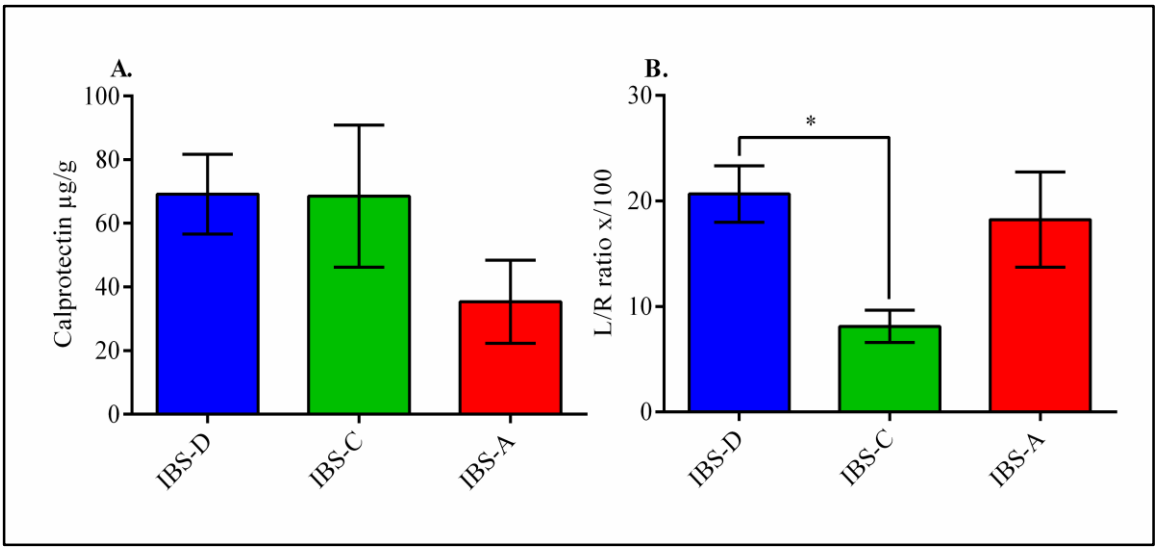

Figure 4.2. Faecal calprotectin and L/R ratio in the different classifications of IBS patients at recruitment. (**A**) Faecal calprotectin levels were not significantly different across the 3 IBS classifications, (**B**) L/R ratio was significantly different in IBS-D patients compared with IBS-C patients at recruitment. L/R ratio in IBS-A patients was elevated compared with levels in IBS-C patients but did not reach significance (p=0.051). IBS-D, diarrhoea dominant (n=20), IBS-C, constipation dominant (n=8) and IBS-A, alternating diarrhoea and constipation (n=6), L/R ratio, lactulose/rhamnose ratio, data shown as mean ± SEM, *p<0.05.

4.5.2 Faecal calprotectin levels in IBS-D patients at recruitment

IBS-D patients were separated from IBS-C and A patients and analysed (Table 4.4). Faecal calprotectin levels ranged from 7.21–169.6 µg/g in IBS-D, mean 69.19 ± 56.10 (Table 4.4) (n=20), with 8 patients >50 µg/g (Table 4.5). Increased *Bifidobacterium* spp. levels were correlated (r=-0.53 (95% CI -0.7948 to -0.1037), p=0.01) with decreased faecal calprotectin levels (Figure 4.3). *F. prausnitzii* was correlated with faecal calprotectin but did not reach significance (r=-0.37, p=0.13) (Figure 4.3). All other bacterium tested showed no association with faecal calprotectin levels.

Table 4-4: Characteristics and measurements in subjects with IBS-D at recruitment. FC, faecal calprotectin (µg/g); L/R, lactulose/rhamnose ratio; SBBO, small bowel bacterial overgrowth; IBS-D, diarrhoea predominant irritable intestine syndrome. Values are shown as mean ± SD.

Patient characteristics	Patient data
Male:Female	6:14
Age in yrs. (mean ± SD)	52.52 ± 17.10
IBS-D	20
L/R ratio (x100) (mean ± SD)	(20.75 ± 13.23)
FC (µg/g) (mean ± SD)	(69.19 ± 56.10)
SBBO (positive H_2 breath test)	2 (1M:1F)
Faeces for RT-PCR	20

Table 4-5: Faecal calprotectin levels of IBS-D patients at recruitment. FC, faecal calprotectin (µg/g). Levels are shown as mean ± SEM

	Number of IBS-D patients	FC, µg/g
FC> 50 µg/g	n=8	116.1 ± 39.89
FC < 50 µg/g	n=10	25.4 ± 12.59

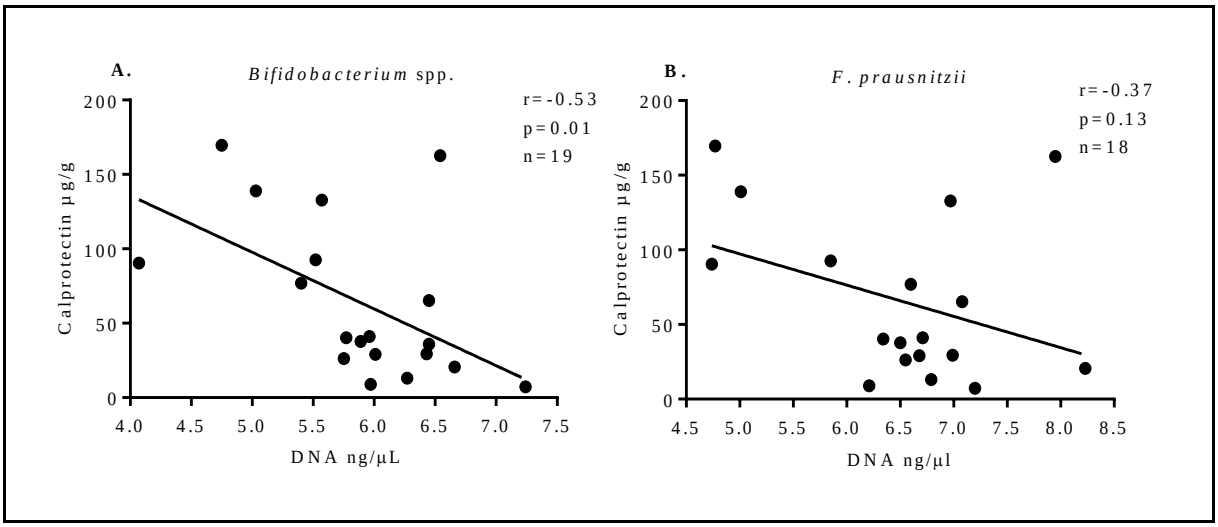

Figure 4.3. Mucosally protective endogenous *Bifidobacterium* spp. and *F. prausnitzii* levels compared with faecal calprotectin levels in IBS-D patients. (**A**) Increased levels of *Bifidobacterium* spp. were correlated with a decrease in faecal calprotectin levels (r=-0.53, p=0.01, n=19) in IBS-D patients. (**B**) Levels of *F. prausnitzii* were correlated (not significant) with faecal calprotectin levels in IBS-D (r=-0.37, p=0.13, n=18).

4.5.3 Faecal calprotectin levels in IBS-C patients at recruitment

IBS-C patients were separated from the general population (n=8) and analysed. Faecal calprotectin levels ranged from 9.6–182.5 µg/g (mean, 68.54 ± 59.08 (SD)). There were no significant correlations with inflammation and any bacteria quantified.

4.5.4 Faecal calprotectin levels in IBS-A patients at recruitment

IBS-A patients were analysed separately from the general population (n=6). Faecal calprotectin levels ranged from 6.00–66.60 µg/g (mean 35.36 ± 29.26 (SD). There were no significant correlations observed between intestinal parameters and bacteria.

4.5.5 Small intestinal permeability in IBS patients at recruitment.

L/R ratio ranged from (2.08–40.86). Mean levels of the L/R ratio in all IBS patients are shown in Table 4.1. Twenty-five patients were >8.8, and 4 patients <8.8 (Table 4.6). Faecal calprotectin and L/R ratio were not correlated at recruitment (r=0.30, p=0.11). A number of associations were seen between intestinal bacteria and small intestinal permeability in the general population of IBS patient in this cohort. *Lactobacillus* spp. were correlated with small intestinal permeability at recruitment (r=-0.52 (95% CI-0.7531 to -0.1935), p=0.003) (Figure 4.4). *Bifidobacterium* spp. were also correlated with intestinal permeability at recruitment (r=-0.59 (95% CI-0.7913 to -0.2679), p=0.001) (Figure 4.4). Small intestinal permeability was correlated with *F. prausnitzii* levels (r=-0.48 (95% CI-0.7304 to -0.1286), p=0.01) (Figure 4.4). *E. coli* levels were also correlated with small intestinal permeability (r=-0.44 (95% CI-0.7028 to -0.07231), p=0.02) (Figure 4.4). There was a significant difference observed between the different classifications of IBS and the L/R ratio. Small

intestinal permeability was significantly different in IBS-D patients compared with levels observed in IBS-C at recruitment (p=0.001) (Figure 4.2).

Table 4-6: L/R ratio in IBS patients at recruitment. L/R ratio is expressed as mean ± SD

	Number of IBS-D patients	L/R ratio (x100) ± SD
L/R ratio > 8.8	n=25	19.72 ± 11.66
L/R ratio < 8.8	n=4	4.85 ± 2.71

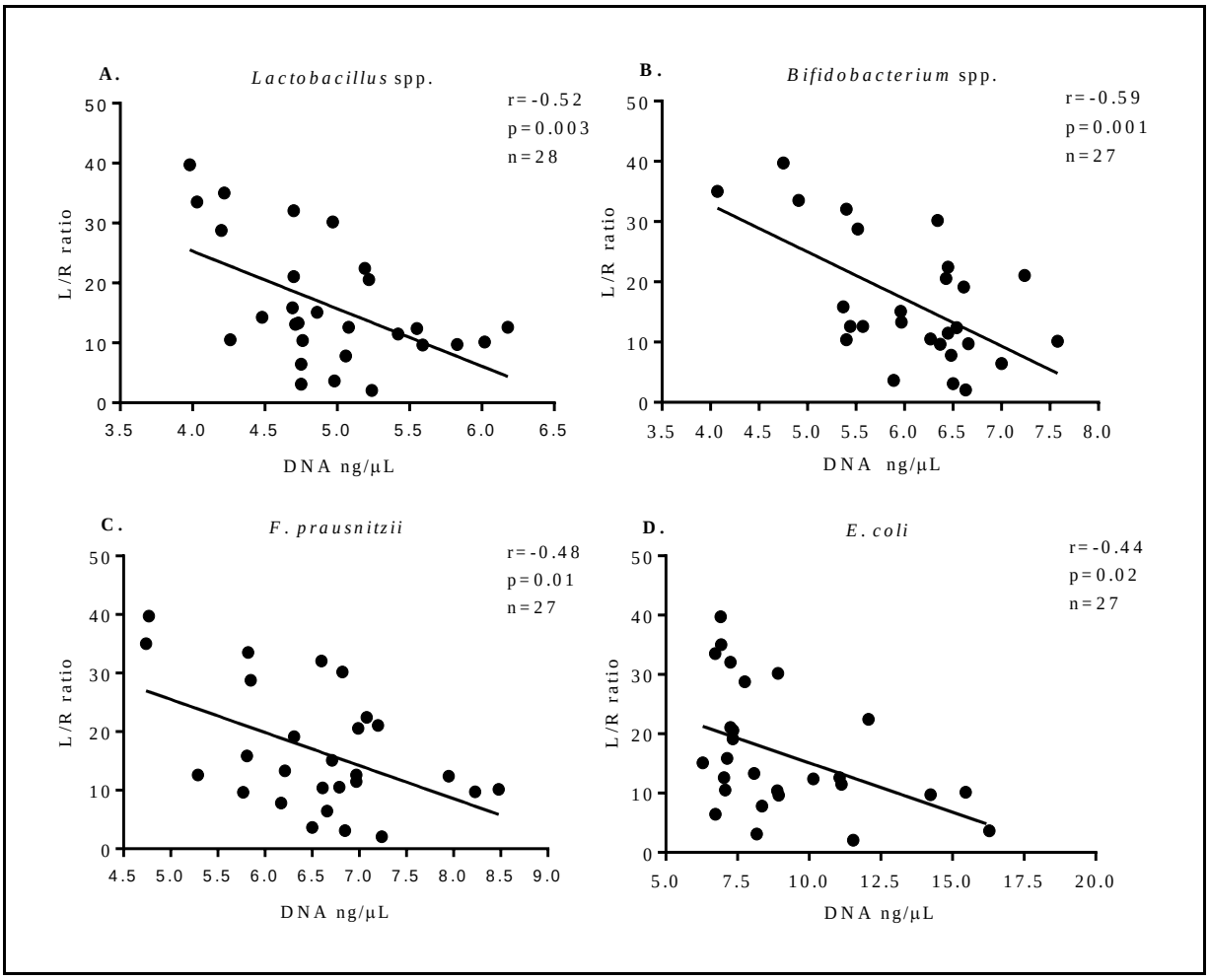

Figure 4.4. Endogenous intestinal bacteria compared with a marker of small intestinal permeability at recruitment in IBS patients. (**A**) *Lactobacillus* spp., were correlated with L/R ratio (r=-0.52 (95% CI-0.7531 to -0.1935), p=0.003), (**B**) *Bifidobacterium* spp. were also correlated with L/R ratio (r=-0.59 (95% CI-0.7913 to -0.2679), p=0.001), (**C**) *F. prausnitzii* were correlated with L/R ratio at recruitment in IBS patients (r=-0.48 (95% CI-0.7304 to -0.1286), p=0.01) and (**D**) *E. coli* levels were also correlated with intestinal permeability in the small intestine at recruitment (r=-0.44 (95% CI-0.7028 to -0.07231), p=0.02).

4.5.6 Small intestinal permeability and the microbiome in IBS-D patients at recruitment.

L/R ratio ranged from (9.72–40.86) in IBS-D patients. 16 patients were >8.8 (9.72–40.86) (Table 4.7). There was a positive correlation between L/R ratio and faecal calprotectin levels (r=0.56 (95% CI 0.05354 to 0.8292), p=0.02) (Figure 4.5). *F. prausnitzii* concentrations were also significantly correlated with decreased intestinal permeability at recruitment (r=-0.70 (95% CI -0.8955 to -0.2605), p=0.006) (Figure 4.6). Increased levels of the endogenous *Bifidobacterium* spp. were significantly correlated with tightening of the mucosal barrier (r=-0.63 (95% CI -0.8596 to -0.20140), p=0.008) (Figure 4.6). *Rosburia* spp. (r=-0.60 (95% CI -0.8461 to -0.1537), p=0.01) and *A. muciniphila* levels (r=-0.52 (95% CI -0.8122 to -0.04601), p=0.04) were also significantly correlated with deceased intestinal permeability respectively (Figure 4.6).

Table 4-7: Lactulose/Rhamnose ratio in IBS-D patients at recruitment. L/R ratio expressed as mean ± SD.

	Number of IBS-D patients	L/R ratio (x100) mean ± SD
L/R ratio > 8.8	n=16	21.72 ± 12.88
L/R ratio < 8.8	n=0	-

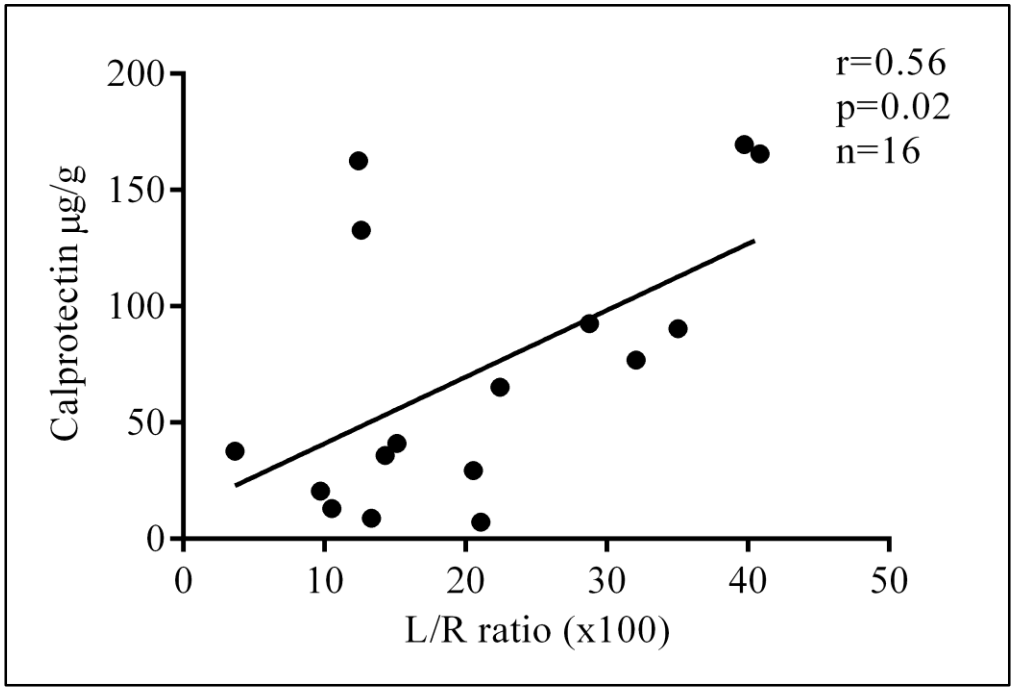

Figure 4.5. Correlations between faecal calprotectin levels and L/R ratio in IBS-D patients. Levels of inflammation and permeability were correlated in IBS-D patients (r=0.55 (95% CI 0.05354 to 0.8292), p<0.03), LR ratio, lactulose/rhamnose ratio.

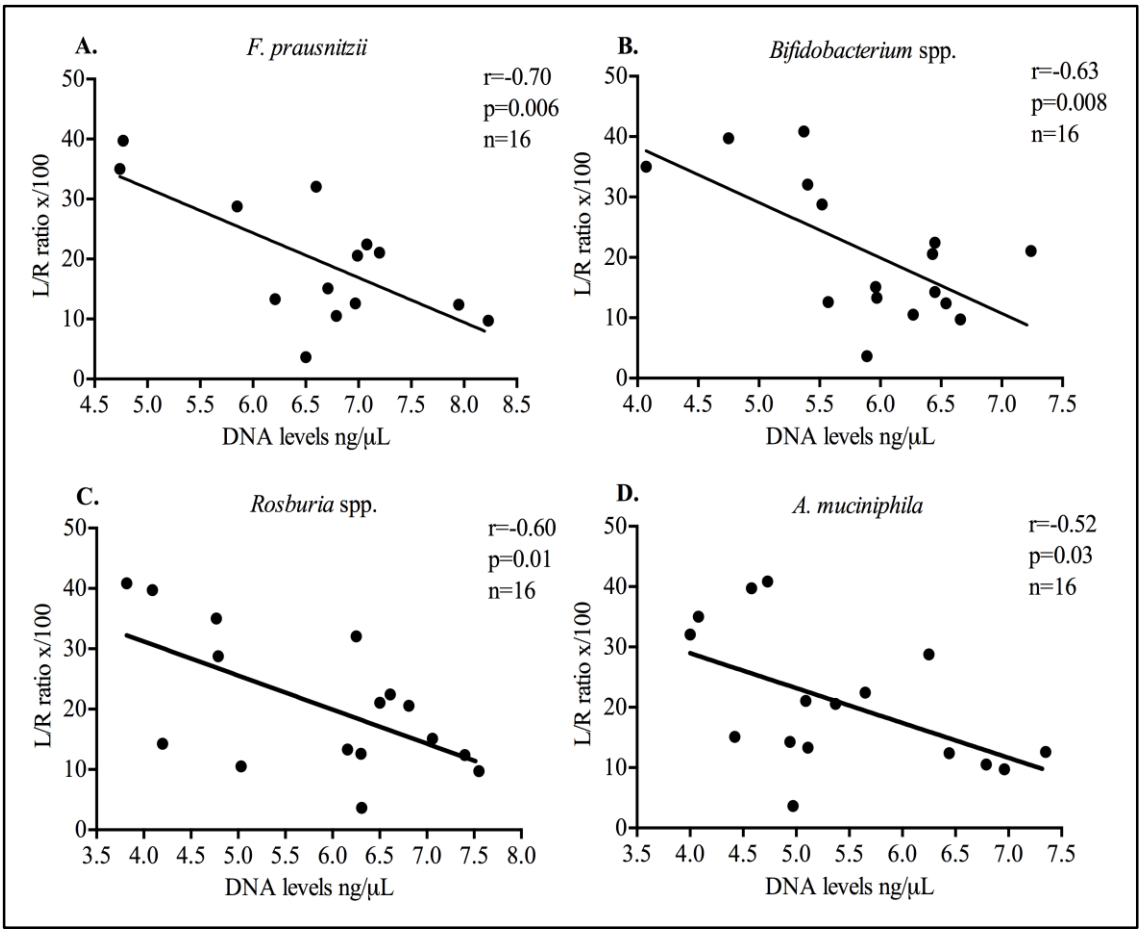

Figure 4.6. Mucosally protective endogenous bacteria compared with L/R ratio in IBS-D patients. (**A**) Increased levels of *F. prausnitzii* were significantly correlated with L/R ratio r=-0.70 (95% CI -0.8955 to -0.2605), p=0.006), (**B**) Increased levels of the endogenously beneficial *Bifidobacterium* spp. were significantly correlated to small intestinal permeability (r=-0.63 (95% CI -0.8596 to -0.20140), p=0.008), (**C**) *Rosburia* spp., a major butyrate producer, was significantly correlated with L/R ratio (r=-0.60 (95% CI -0.8461 to -0.1537), p=0.01 and (**D**) Mucosally associated endogenous commensal, *A. muciniphila,* were correlated with tightening of the small intestinal mucosal barrier in IBS-D patients (r=-0.52 (95% CI -0.8122 to -0.04601), p=0.04).

4.5.7 Small intestinal permeability and the microbiome in IBS-C patients at recruitment

At recruitment 8 patients of the general population of IBS patients had constipation as the dominant symptom. L/R ratio in these patients ranged from 2.08–12.61 (mean 8.12 ± 4.74 (SD)) No significant correlations were observed between bacteria and L/R ratio at recruitment in the IBS-C group.

4.5.8 Small intestinal permeability and the microbiome in IBS-A patients at recruitment

There were 6 patients in the IBS-A group. The L/R ratio in this group ranged from 6.43 to 33.50 (mean 18.24 ± 11.03 (SD)). There were no significant correlations between bacteria and L/R ratio at recruitment in this category of IBS patients.

4.6 Discussion

In this retrospective analysis of faecal samples from a cohort of IBS patients positive associations were recorded between endogenous beneficial bacteria and non-invasive markers of mucosal stress. This cohort is remarkably different from other cohorts of IBS sufferers reported in the literature as less than 10% of all patients enrolled in the current study had SBBO at recruitment. Others have reported SBBO rates of between 50-80% in IBS populations (Pimentel, Chow and Lin 2000). There was a significant correlation with the small intestinal permeability and faecal calprotectin levels in the general IBS cohort. There were also some significant correlations observed in the different classifications of IBS. This data may suggest that these two non-invasive tests, when used in combination, may be a potential diagnostic for managing IBS-D patients compared with the general IBS population. There were also correlations observed between endogenous beneficial bacteria of the microbiome and non-invasive markers of mucosal stress.

It must be stated that IBS is believed to be a low-grade inflammation and has subsets with an altered small intestinal permeability. However, these parameters have been infrequently examined in concert with, and also in relation to alterations in the faecal microbiome. The data presented here contrasts IBS patients regardless of their predominant symptom classification and subsequently examines each subset of IBS categorization.

F. prausnitzii is an endogenous putatively known beneficial bacteria of the intestinal microbiome, with reported anti-inflammatory actions. In this study increased levels *F. prausnitzii* showed a strong association with decreased small intestinal permeability in both

the general IBS population and in IBS-D patients. Recently, *F. prausnitzii* has shown many beneficial attributes in relation to promoting GIT functioning such as decreasing inflammation and reducing intestinal permeability (Carlsson, Yakymenko, Olivier *et al.* 2013). Studies on IBD patients have reported this bacterium to be lower in CD patients compared to controls (Sokol, Seksik, Furet *et al.* 2009, Willing, Halfvarson, Dicksved *et al.* 2009). Indeed, increased levels of *F. prausnitzii* have been associated with longer remission periods in Crohn's disease (CD) (Sokol, Seksik, Furet *et al.* 2009). A major butyrate producer within the colon, *F. prausnitzii* represents between 5% and up to 15% of the total bacteria in some individuals (Sokol, Thomas, Wells *et al.* 2013). A recent study using a DSS induced colitis mouse model found *F. prausnitzii* supernatant decreased intestinal permeability, measured by the passage of ^{51}Cr-EDTA across the intestinal membrane in Ussing chambers compared to controls (Carlsson, Yakymenko, Olivier *et al.* 2013). Others have reported the supernatant of *F. prausnitzii* had anti-inflammatory effects in a trinitrobenzenesulponic acid (TNBS) induced colitis model in mice (Sokol, Pigneur, Watterlot *et al.* 2008), however, the mechanism of action and the components produced by *F. prausnitzii* are as yet not clearly defined (Ricci, Girosi, Tarr *et al.* 2006). The data from the current analyses suggests that levels of *F. prausnitzii* may promote tightening of the mucosal barrier in the small intestine, preventing luminal contents from traversing the mucosal barrier triggering an immune response leading to inflammation.

The relationship between *F. prausnitzii* and decreased intestinal permeability also fit the association observed between faecal calprotectin levels and L/R ratio in this cohort of IBS patients. Recently, *F. prausnitzii* and *F. prausnitzii* supernatant were shown to reduce inflammation in a chronic low grade inflammatory mouse model induced by dinitro-benzene

sulfonic acid (DNBS) (Martín, Miquel, Chain *et al.* 2015). The authors reported significantly reduced levels of IL-6, INF-γ, IL-4 in colonic contents, and significantly reduced levels of IL-6 and IL-22 in serum of treated mice compared to DNBS mice without *F. prausnitzii* and *F. prausnitzii* supernatant (Martín, Miquel, Chain *et al.* 2015). Intestinal permeability was also measured *in vivo* and *in vitro*. *In vivo* measurements of permeability were made by fluoroscein-conjugated dextran tracer (FITC-dextran) and quantified in blood, while *in vitro* assessment of colonic tissue was made using ^{51}Cr-EDTA flux, in Ussing chambers. Both methods showed statistically significant reduction in intestinal permeability in the treatment group compared with controls (Martín, Miquel, Chain *et al.* 2015). These results confirm other reports of the anti-inflammatory capabilities and the mucosally protective attributes of *F. prausnitzii.* To the authors knowledge there has not been any report of faecal levels of *F. prausnitzii* and an association with intestinal permeability as measured with the L/R ratio in a cohort of IBS patients. Well-designed studies are now required to investigate the benefits of this large commensal group within the colon and its possible remote effects on promoting barrier function by decreasing intestinal permeability in the small intestinal.

Correlations were also recorded between *F. prausnitzii, Bifidobacterium* spp. and *Rosburia* spp. (Appendix 4.1). *F. prausnitzii* and *Rosburia* spp. are major butyrate producers in the colon whereas *Bifidobacterium* spp. are unable to produce butyrate (Scott, Martin, Duncan *et al.* 2014) but are associated with a known butyrogenic effect (Falony, Vlachou, Verbrugghe *et al.* 2006, Rivière, Gagnon, Weckx *et al.* 2015). These correlations suggest that *Bifidobacterium* spp., *F. prausnitzii* and *Rosburia* spp. may function syntrophically to produce butyrate and other undefined metabolites, which may affect the luminal and mucosal environment, to decrease inflammation and tighten barrier function in IBS-D.

Certain *Bifidobacterium* spp. have also been shown to aid mucosal repair in a stressed mucosal model in rats by producing bifidobacteria adhesion (BA). BA is a protein secreted by *B. adolescentis* and resulted in decreased mucosal stress and lead to mucosal repair (Shu, Yu, Kang *et al.* 2014). The strong correlation between *Bifidobacterium* spp., *F. prausnitzii* and *Rosburia* spp. may produce a syntrophism in which the production of butyrate and other metabolites beneficially affect parameters of intestinal heath such as intestinal permeability and inflammation.

Interestingly, *E. coli* levels were associated with tightening of the small intestinal mucosa in the general IBS population. However, this was not observed in the separate IBS categories. *E. coli* are a known commensal microbe within the intestinal tract of humans, and are one of the first colonizers of the human GIT (Rotimi and Duerden 1981). Although *E. coli* strains vary considerably in pathogenicity, the strain diversity was not undertaken in this study. Indeed the non-pathogenic probiotic strain *E. coli* Nissle 1917 has been studied as a probiotic and prevents the adhesion of a variety of pathogens to human embryonic intestinal epithelial INT407 cells *in vitro* (Altenhoefer, Oswald, Sonnenborn *et al.* 2004). Also other studies have shown that *E. coli* Nissle 1917 can dampen the inflammatory response by suppressing TNF-α activation of IL-8 (Kamada, Maeda, Inoue *et al.* 2008). An *in vivo* study in an animal model of DSS induced colitis in mice showed *E. coli* Nissle 1917 was able to protect the intestinal barrier and tighten the mucosal barrier as shown using an Ussing chamber (Ukena, Singh, Dringenberg *et al.* 2007). Although, the endogenous strains and potential attributes of *E. coli* in this study are unknown, it is possible that specific endogenous strains of *E. coli* may enhance barrier function leading to a more patent small intestinal mucosa.

Higher levels of mucosally protective endogenous *Lactobacillus* spp. and *Bifidobacterium* spp. appear to be associated with a higher degree of tight junctional patency in IBS-D patients. This has been determined for the small intestine using the dual sugar permeability test and may or may not be indicative of tight junction patency throughout the GIT. *Lactobacillus* and *Bifidobacterium* spp. have both shown positive attributes in regards to GIT health (Turroni, Ventura, Buttó *et al.* 2014).

A. muciniphila in a number of studies has shown beneficial attributes in relation to the mucosa. *A. muciniphila* utilizes mucins as a sole carbon source, producing acetate and propionate. Both acetate and propionate have shown anti-inflammatory properties in the GIT (Tedelind, Westberg, Kjerrulf *et al.* 2007). Furthermore, *A. muciniphila* also liberates oligosaccharides from mucins, providing substrate for the production of short chain fatty acids and other metabolites, which may stimulate host responses directly benefiting the mucosa (Belzer and De Vos 2012). This study has shown a link between the mucosally associated *A. muciniphila* and the L/R ratio. Studies have reported decreased levels of *A. muciniphila* in IBD patients and patients with appendicitis compared with healthy controls (Belzer and De Vos 2012). Interestingly, de Vos *et al* 2011, found *A. muciniphila* was associated with an increase in L cells (Everard, Lazarevic, Derrien *et al.* 2011). L cells secrete glucagon like peptide-1 and 2 (GLP)-1 and -2), which have been shown to directly affect barrier function by decreasing intestinal permeability. *A. muciniphila* has also been shown to increase mucin turnover and elevate levels of endocannabinoids which control inflammation, gut peptide secretion and affect intestinal permeability (Everard, Belzer, Geurts *et al.* 2013).

In a recent study of gut inflammation and food intolerance, raised levels of faecal calprotectin correlated with decreased bacterial abundance (Hippe, Remely, Bartosiewicz *et al.* 2014). Total bacterial abundance was correlated with *F. prausnitzii* and *A. muciniphila* levels, indicating that these bacteria may be responsible for reduced inflammation in the GIT. The authors also reported that increased bacterial abundance was associated with decreased abdominal pain compared to low bacterial abundance in these patients (Hippe, Remely, Bartosiewicz *et al.* 2014).

The reported correlations in this study confirm what others have reported, in that *F. prausnitzii, A. muciniphila*, and endogenous *Lactobacillus* spp. and *Bifidobacterium* spp. are mucosally protective and may play a role in mediating inflammation and intestinal permeability in the GIT. The data is suggestive of a beneficial role of these endogenous beneficial bacteria in reducing mucosal stress in IBS patients. Furthermore, the data suggests that faecal levels of endogenous bacteria combined with non-invasive markers of mucosal dysfunction may help delineate between different categories of IBS.

4.7 Conclusions

This retrospective analysis was undertaken to determine the associations of specific endogenous bacteria associated with a healthy mucosa and parameters indicative of altered gut health in patients with IBS. In summary, this study has quantitatively shown associations between known beneficial endogenous bacteria and endogenous bacteria associated with a healthy mucosa and non-invasive markers of mucosal stress. The intestinal microbiota is a diverse ecosystem with much synergism existing between microbes. The correlations reported in the current study between specific beneficial microbes and parameters of mucosal stress might be useful as potential indicators of mucosal stress in certain phenotypes of IBS. Rigorous prospective trials to test these findings are warranted in light of the now widely accepted role of increased intestinal permeability and chronic low-grade mucosal inflammation, and more broadly mucosal stress in IBS patients. Unravelling the interactions of the microbial environment and determining the role of these endogenous microorganisms are urgently needed. Furthermore, understanding the relationship of the microbes in relation to markers of mucosal stress may provide therapeutic options for the treatment of stressed mucosa in IBS patients.

Appendix 4.1. Correlation matrix of all bacteria and intestinal parameters at recruitment in IBS patients. LR, lactulose /rhamnose ratio, SRB, sulphate reducing bacterial, n=34

	Bifidobacterium spp.	*Lactobacillus* spp.	*F. prausnitzii*	*A. muciniphila*	*E. coli*	*Rosburia* spp.	*R. bromi*	*R. gnavus*	*R. torques*	L/R ratio	Calprotectin
Bifidobacterium spp.	1										
Lactobacillus spp.	0.54	1									
F. prausnitzii	0.79	0.65	1								
A. *muciniphila*	0.37	0.45	0.52	1							
E. coli	0.11	0.05	0.06	-0.16	1						
Rosburia spp.	0.46	0.66	0.65	0.33	-0.13	1					
R. bromi	0.50	0.30	0.36	0.30	-0.12	0.28	1				
R. gnavus	0.43	0.64	0.57	0.10	0.04	0.64	0.31	1			
R. torques	0.35	0.38	0.41	0.38	-0.24	0.35	0.39	0.37	1		
L/R ratio	-0.59	-0.53	-0.48	-0.26	-0.28	-0.34	-0.28	-0.23	-0.03	1	
Calprotectin	-0.39	0.01	-0.42	-0.02	0.00	-0.22	-0.36	-0.11	-0.04	0.18	1

Appendix 4.2 Correlational matrix of all bacteria and intestinal parameters in IBS-D patients at recruitment. LR, lactulose /rhamnose ratio, SRB, sulphate reducing bacteria, n=20

	Bifidobacterium spp.	*Lactobacillus* spp.	*F. prausnitzii*	*A. muciniphila*	*Rosburia* spp.	*R. bromi*	*R. gnavus*	*R. torques*	*E. coli*	L/R ratio	Calprotectin
Bifidobacterium spp.	1										
Lactobacillus spp.	0.45	1									
F. prausnitzii	0.45	0.55	1								
A. muciniphila	0.82	0.76	0.51	1							
Rosburia spp.	0.54	0.77	0.27	0.78	1						
R. bromi	0.43	0.27	0.13	0.20	0.29	1					
R. gnavus	0.14	0.63	0.02	0.52	0.74	0.23	1				
R. torques	0.24	0.50	0.39	0.23	0.31	0.37	0.07	1			
E. coli	0.31	0.46	0.29	0.43	0.18	0.12	0.41	0.13	1		
L/R ratio	-0.62	-0.56	-0.49	-0.68	-0.50	-0.28	-0.43	-0.08	-0.58	1	
Calprotectin	-0.10	-0.01	0.31	-0.25	-0.24	0.20	-0.25	0.07	-0.12	0.23	1

Appendix 4.2 Correlational matrix of all bacteria and intestinal parameters in IBS-C patients at recruitment. LR, lactulose /rhamnose ratio, SRB, sulphate reducing bacteria, n=8

	Bifidobacterium spp.	*Lactobacillus* spp.	*F. prausnitzii*	*A. muciniphila*	*Rosburia* spp.	*R. bromi*	*R. gnavus*	*R. torques*	*E. coli*	L/R ratio	Calprotectin
Bifidobacterium spp.	1										
Lactobacillus spp.	0.62	1									
F. prausnitzii	0.90	0.51	1								
A. muciniphila	0.49	0.28	0.74	1							
Rosburia spp.	0.43	0.17	0.73	0.92	1						
R. bromi	0.90	0.51	1.00	0.74	0.73	1					
R. gnavus	0.77	0.89	0.72	0.39	0.43	0.72	1				
R. torques	0.49	0.35	0.61	0.61	0.79	0.61	0.64	1			
E. coli	0.81	0.74	0.77	0.51	0.50	0.77	0.84	0.71	1		
L/R ratio	-0.18	0.36	-0.31	-0.21	-0.13	-0.31	0.31	0.32	0.24	1	
Calprotectin	-0.84	-0.33	-0.60	-0.47	-0.42	-0.80	-0.43	-0.08	-0.37	0.61	1

Appendix 4.3 Correlational matrix of all bacteria and intestinal parameters in IBS-A patients at recruitment. LR, lactulose /rhamnose ratio, SRB, sulphate reducing bacteria, n=6

	Bifidobacterium spp.	*Lactobacillus* spp.	*F. prausnitzii*	*A. muciniphila*	*Rosburia* spp.	*R. bromi*	*R. gnavus*	*R. torques*	*E. coli*	L/R ratio	Calprotectin
Bifidobacterium spp.	1										
Lactobacillus spp.	0.61	1									
F. prausnitzii	0.76	0.52	1								
A. muciniphila	-0.61	-0.84	-0.37	1							
Rosburia spp.	0.27	0.74	-0.04	-0.81	1						
R. bromi	0.73	0.09	0.50	-0.37	-0.15	1					
R. gnavus	0.79	0.85	0.55	-0.79	0.75	0.25	1				
R. torques	0.77	0.47	0.82	-0.17	0.01	0.29	0.64	1			
E. coli	0.34	0.50	0.84	-0.18	-0.04	-0.01	0.35	0.66	1		
L/R ratio	-0.47	-0.46	-0.36	0.57	-0.08	-0.64	-0.18	-0.02	-0.12	1	
Calprotectin	0.57	0.44	-0.02	-0.35	0.38	0.31	0.41	0.39	-0.40	-0.48	1

Chapter 5.0 The hydrogen economy in patients with IBS related to non-invasive markers indicative of a stressed mucosa

5.1 Preface

The governance of the hydrogen (H_2) produced through the fermentative activity of the microbiome is referred to as the hydrogen economy. Bloating, visceral pain and altered bowel habits are the dominant symptoms of IBS and are probably indicative of mucosal stress. The regulation of intestinal gases may play a role in the aforementioned symptoms, and are important to understand in the context of possible interventions to decrease or eliminate symptoms related to mucosal stress. This chapter examines for the first time the relationship between intestinal gases (H_2 and CH_4), the dominant microorganisms that control them, and non-invasive markers of mucosal stress in a well classified cohort of IBS patients. This study has shown gases indicative of fermentative activity of the intestinal microbiome may have a potential role in increasing small intestinal patency and modulating intestinal inflammation in certain classifications of IBS. This study has also shown that the two dominant hydrogenotrophs were significantly correlated with each other and that this may be a microbial indicator of IBS. Moreover, this data suggests that the fermentative activity of the microbiome may have a possible diagnostic role in certain subsets of IBS.

5.2 Abstract

Introduction

IBS is the most widely diagnosed functional gastrointestinal illness in the world occurring in up to 20% of the population. Symptoms of IBS related to bloating are some of the most distressing experienced by IBS patients. Control of fermentative gases and the relationship to the mucosa under stress are emerging as possible contributors to mucosal dysfunction.

Materials and methods

This retrospective study quantitated faecal levels of specific bacteria utilizing PCR, and compared them with parameters of mucosal stress and breath levels of CH_4 and H_2 in a cohort of IBS patients.

Results

In this retrospective analysis the most significant findings are that H_2 levels measured in breath in non-CH_4 excretors were significantly correlated with the tightening of the small intestinal mucosa as measured by the L/R ratio ($r=-0.75$, $p=0.008$). Levels of faecal calprotectin measured in stool were significantly lower ($p=0.03$) in CH_4 excretors compared with non-excretors. Levels of faecal calprotectin were also significantly and positively correlated with CH_4 levels in breath of CH_4 excretors ($r=0.60$, $p=0.02$). There was also a highly significant positive relationship between the two major hydrogenotrophic microorganisms ($r=0.73$, $p=0.002$). This study has shown associations with specific non-invasive markers of mucosal stress and fermentative activity of the intestinal microbiome measured in breath related to the H_2 economy.

Conclusions

These findings may be useful as functional markers of stressed mucosa in subsets of IBS patients. These associations need to be investigated in light of these findings as they may aid in the identification of stressed mucosa in certain subsets of IBS patients.

5.3 Introduction

Early studies in irritable bowel syndrome (IBS) suggested marked differences in the activity of fermentation in this syndrome (King, Elia and Hunter 1998). The relationship between this, symptoms, and the composition of the faecal microbiome is only now beginning to be investigated. IBS is an increasingly common FGID in industrialised nations (Palframan, Gibson and Rastall 2003, Halvorson, Schlett and Riddle 2006, Spiller 2007, Salonen, De Vos and Palva 2010). No clear aetiological precursors are present. However, emerging evidence suggests compositional changes in the microbiome and related metabolites, lead to mucosal dysfunction that plays a causative role in disease pathogenesis and symptomatology (Jeffery, O'toole, Öhman *et al.* 2012). Organic changes, such as low-grade mucosal inflammation and altered intestinal permeability indicate a degree of mucosal dysregulation which may be related to compositional shifts in the microbiota (Rajilić–Stojanović, Biagi, Heilig *et al*. 2011). Altered patterns in microbial composition may modify the metabolic output of the microbiome, eliciting distinct host responses via mucosal disruption. IBS presents with a complex symptomatology including chronic abdominal pain, bloating, distension and variable bowel habits (Tana, Umesaki, Imaoka *et al*. 2010). Fermentation products of the microbiota such as hydrogen (H_2) and methane (CH_4) may contribute to the aforementioned symptoms. For example, expired CH_4 levels have been documented in constipated IBS patients (Chatterjee, Park, Low *et al*. 2007, Attaluri, Jackson, Valestin *et al.* 2010). Reduction of microbial diversity has also been associated with a variety of disorders, such as diabetes, immunoallergenic conditions such as allergy and atopic disease and low grade inflammatory syndromes such as IBS when compared to healthy controls (Isolauri 2012, Shanahan 2012). It still remains to be shown if the microbiota and there fermentative products is the cause or consequence of altered motility, permeability and secretion (Sanders, Guarner, Guerrant *et al.* 2013).

The human GIT is inhabited by trillions of microorganisms from diverse taxa, with a vast genome that has only recently been acknowledged as fundamentally important for human health (Qin, Li, Cai *et al*. 2012, Lepage, Leclerc, Joossens *et al*. 2013). Indeed, the GIT can be thought of as bioreactor with discrete compartments performing specific tasks. Absorption, digestion and assimilation occur along the length of the small intestine, while water absorption and fermentation of substrates occurs in the slower moving environment of the colon. Colonic fermentation by the resident microbiota is the only source of H_2 produced by humans. Management of colonic H_2 is known as the H_2 economy, and is partly governed by hydrogenotrophs such as methanogenic archaea and sulphate-reducing bacteria (SRB). By cycling a range of elements and metabolites, hydrogenotrophs are responsible in part for orchestrating digestive biochemistry (Carbonero, Benefiel and Gaskins 2012). Removal of H_2 and other products of fermentation, result in the production of CH_4, hydrogen sulphide (H_2S) and acetate. Breath CH_4 has been detected in varying amounts ranging from 34% to 87% in human populations and differs according to ethnicity (Levitt, Furne, Kuskowski *et al*. 2006). A small proportion of the population do not produce enough H_2 and CH_4 to be detected in breath, but nonetheless harbour fermentative bacteria and methanogens. However, using molecular methods, methanogenic archaea and SRB can be detected in stool in almost 99% of the population (Dridi, Henry, El Khéchine *et al*. 2009, Carbonero, Benefiel, Alizadeh-Ghamsari *et al.* 2012).

SRBs are a group of microorganisms that utilise sulphate as the terminal electron acceptor for respiration, and produce H_2S in the human colon (Carbonero, Benefiel and Gaskins 2012). SRBs are ubiquitous within the human colon and have been detected in the intestinal mucosa

(Kleessen, Kroesen, Buhr *et al*. 2002), and in human stool in the vicinity of 10^3–10^{11} cells/g of faeces (Gibson, Macfarlane and Cummings 1988, Carbonero, Benefiel and Gaskins 2012). H_2S production occurs both at a cellular level in many sites throughout the body, and in the GIT from microbial activity. Extracellular production of H_2S occurs in the GIT by the reduction of dietary sulphate and derived from intestinal mucin to H_2S by SRBs. Recently, a role for SRBs in the development of IBS has been suggested as increased levels of H_2S may play a role in symptom development. Using molecular methods (PCR), increased levels of SRBs and H_2S were detected in faeces in a cohort of IBS-C patients compared with levels detected in healthy controls (Chassard, Dapoigny, Scott *et al*. 2012). The role of SRBs is as yet to be clearly elucidated in regards to the links with certain gastrointestinal illnesses and their potential role in gastrointestinal health.

Of the many symptoms in IBS, abdominal distension, bloating and visceral hypersensitivity to pain are among the most distressing and clinically relevant. In the absence of well-defined biomarkers, diagnosis of IBS is characterised by a clearly outlined specific symptom criteria (ROME 111). Bloating and hypersensitivity to pain may arise from an excess of fermentative gases such as H_2, CH_4 and CO_2, directly affecting the mucosa. End products of fermentation interact with and benefit the colonic mucosa, as is the case of SCFAs. To date, many of the products of fermentation are ill- defined, and their effects on the intestinal mucosa are poorly understood. Furthermore, it has become increasingly apparent that the microbiome produces a vast array of metabolites and waste gases that interact with the intestinal mucosa. The interplay of these metabolites appears bidirectional, affecting the enteric nervous system with implications for an array of host responses, such as mood alteration and GIT motility. There

is paucity of information regarding the role of these metabolites in the homeostatic functioning of the GIT (Egert, De Graaf, Smidt *et al.* 2006). Many will undoubtedly affect intestinal permeability and motility, while others will initiate or combat inflammatory pathways. Presently, the most common bacterial products that can be easily measured non-invasively are the intestinal gases H_2, CH_4 and CO_2. There are currently no objective or functional biomarkers of IBS routinely used in the management and the diagnosis of IBS. The aim of this study was to determine the relationship between non-invasive measures of microbial activity H_2 and CH_4, the microorganisms that significantly influence the H_2 economy in the lumen, and functional markers of mucosal stress in a cohort of IBS patients.

5.4 Materials and Methods

The analyses in the present retrospective study were in baseline (pre-randomisation) data (bacterial levels from 1 stool sample at enrolment, intestinal permeability test conducted at enrolment (Lactulose rhamnose ratio), faecal calprotectin levels in faecal samples at enrolment and breath hydrogen and methane levels over 180 mins at enrolment) of samples collected from participants of a double blind, randomised placebo controlled crossover trial, designed to investigate the potential anti-inflammatory benefits of Lyprinol (a green-lipped mussel extract) in the treatment of IBS, as previously described (Chapter 4, Section 4.4.1 page 136 and Table 4.1, page 137).

5.4.1 Small intestinal permeability testing

Intestinal permeability was measured using the Lactulose/Rhamnose (L/R) ratio in blood, as described in Chapter 4, 4.4.2, page 141.

5.4.2 Faecal calprotectin

Stool samples were stored at -70°C until analysed. Quantitative lateral flow assay was used to assess concentrations of faecal calprotectin (Bühlmann Laboratories AG, Switzerland), as described in Chapter 4, 4.4.3, page 141.

5.4.3 Breath testing

Testing for SBBO was undertaken by using a combined H_2 breath test and dual sugar test for small intestinal permeability (Ritchie, Brewster, Davidson *et al.* 2009), as described in Chapter 4, 4.4.4, page 141.

5.4.4 DNA extraction

DNA extraction DNA was extracted from 0.25 g of faeces using the bead beating plus column method described by Morrison and Yu, as described in Chapter 4, 4.4.5, page 142.

5.4.5 Quantitative real-time PCR

All qPCR analyses were performed on a CFX 384TM real-time PCR (RT-PCR) detection system (Bio-Rad, Hercules, CA) in triplicate in a total volume of 10 µL, as described in Chapter 4, 4.4.6, page 136. SRBs were quantitated using primers targeting the dissimilatory sulfite reductase gene (Dsr) (Table 5.1). The methyl coenzyme-M reductase A (mcrA) gene was used to target methanogens in this study (Table 5.1). Amplification of the methanogen

primers was done by a 2 step thermal cycling protocol consisting of initial denaturation step of 50°C for 2 min and 95°C for 2 min followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. The PCR cycling conditions for SRBs were as follows: hotstart at 98°C for 3 min followed by 35 cycles of 2-step PCR with denaturing at 98°C for 15 s and annealing/elongation time and temperature as given below (Table 5.1). Data was analysed with Bio-Rad CFX Manager software (version 1.5) for absolute quantities bacterial levels are reported in ng/µL. Data were normalized by log10 transformation.

Table 5-1: Primers used in this study.

Target gene	Primer sequence	Annealing temp/time	Product size
mcrA (Denman, Tomkins and Mcsweeney 2007)	qmcrA-F, 5' TTC GGT GGA TCD CAR AGR GC qmcrA-R, 5' GBA RGT CGW AWC CGT AGA ATC C	60°C, 60 s	140
DsrA (Nakamura, Leigh, Mackie *et al*. 2009)	Dsr1 F; ACS CAC TGG AAG CAC GGC GG Dsr1R; GTG GMR CCG TGC AKR TTG G	63°C, 20 s	221

5.4.6 Statistical analysis

Statistical analysis of data from stool analysis, breath testing and permeability testing was carried out using Graphpad Prism 6 software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). Pearson's correlation and Spearman's correlation were used to determine changes between intestinal parameters and bacterial levels. Normality of data was tested using D'Agostino & Pearson omnibus normality test. Non-parametric data was analysed using the Mann Whitney U test, with Bonferroni test used to determine significance of p values. $P < 0.05$ was considered as significant. Data are expressed as mean ± SEM.

5.5 Results

Data presented in this chapter is the quantification of faecal levels of hydrogenotrophic microbes from IBS patients classified by the ROME II criteria, and presented in three distinct sections, A, B and C. Section A, is presented as the combined cohort irrespective of gas type and phenotype. Section B, is the cohort separated into gas type (CH_4 excretors and non-CH_4 excretors). Section C, is the cohort separated according to IBS phenotypes, IBS-D, C and A. The most interesting findings of this study were the significant associations between faecal levels of hydrogenotrophic microorganisms, with non-invasive markers of mucosal stress and altered levels of intestinal gases (H_2 and CH_4) detected in breath.

5.5.1 Section A) All IBS categories at recruitment

Significant associations were observed between microorganisms controlling the H_2 economy, non-invasive indices of mucosal health and intestinal gases measured in breath (Table 5.2). The dominant hydrogenotrophic microorganisms were strongly correlated with each other in all IBS phenotypes (r=0.73 (95% CI 0.5076 to 0.8576), p<0.0001) (Figure 5.1). Faecal calprotectin levels was correlated with faecal methanogens (r=-0.41 (95% CI (95% CI-0.7141 to -0.1532), p=0.02) (Figure 5.2). Higher faecal levels of SRBs detected using PCR were correlated with intestinal inflammation in all phenotypes (r=-0.39 (95% CI -0.6561 to -0.07005), p=0.02) (Figure 5.2). Faecal methanogens were not correlated with H_2 breath levels (r=-0.10, p=0.6), but were, as expected, correlated with expired CH_4 on breath (r=-0.38 (95% CI 0.0250 to 0.6571), p=0.03) (figure 5.3). SRB levels were correlated to CH_4 levels (r=0.47 (95% CI 0.1469 to 0.7150), p=0.03) (Figure 5.3), but not with breath H_2 levels (r=-0.31, p=0.08).

Expired H_2 levels in breath were correlated with levels of breath CH_4 (r=-0.47 (95% CI -0.7109 to -0.1257), p=0.007) (Table 5.2). H_2 levels did not correlate with L/R ratio or faecal calprotectin levels (r=-0.27, p=0.2 and r=-0.02, p=0.9 respectively) (Table 5.2). Breath CH_4 levels were not correlated with intestinal permeability (r=-0.23, p=0.3), but were weakly correlated (not significant) with levels of faecal calprotectin (r=-0.36 (95% CI -0.6441 to 0.0115), p=0.05) (Table 5.2).

Table 5-2: Correlational matrix for hydrogenotrophic microorganisms, intestinal gases and intestinal parameters of mucosal stress. IBS patients (n=34), H_2, hydrogen; CH_4, methane; SRB, sulphate reducing bacteria; FC, faecal calprotectin (µg/g); L/R ratio, lactulose/rhamnose ratio. H_2 and CH_4 were measured in breath and expressed as parts per million (ppm).

	Faecal SRB	Faecal methanogens	Breath H_2 (ppm)	Breath CH_4 (ppm)	L/R ratio	FC
Faecal SRB	1					
Faecal methanogens	0.73	1				
Breath H_2 (ppm)	-0.31	-0.10	1			
Breath CH_4 (ppm)	0.47	-0.38	-0.47	1		
L/R ratio	-0.32	-0.17	-0.27	-0.23	1	
FC	-0.39	-0.41	-0.02	-0.36	0.30	1

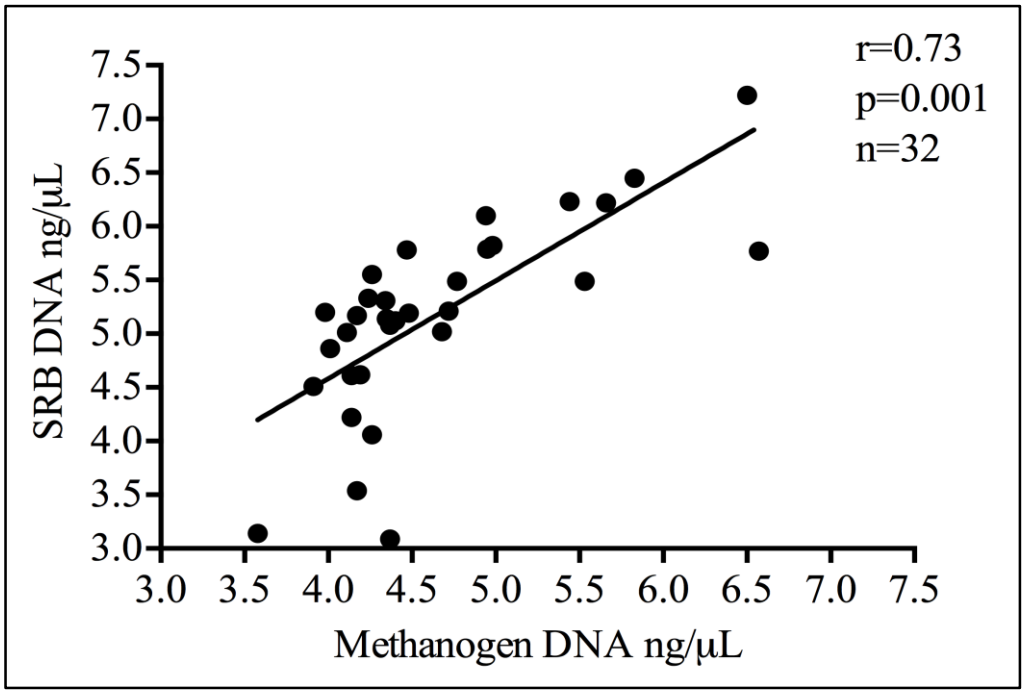

Figure 5.1. Faecal methanogen DNA levels compared to faecal SRB DNA levels in all IBS phenotypes. Both hydrogenotrophic bacteria were highly correlated with each other (r=0.73 (95% CI 0.5076 to 0.8576), p<0.0001).

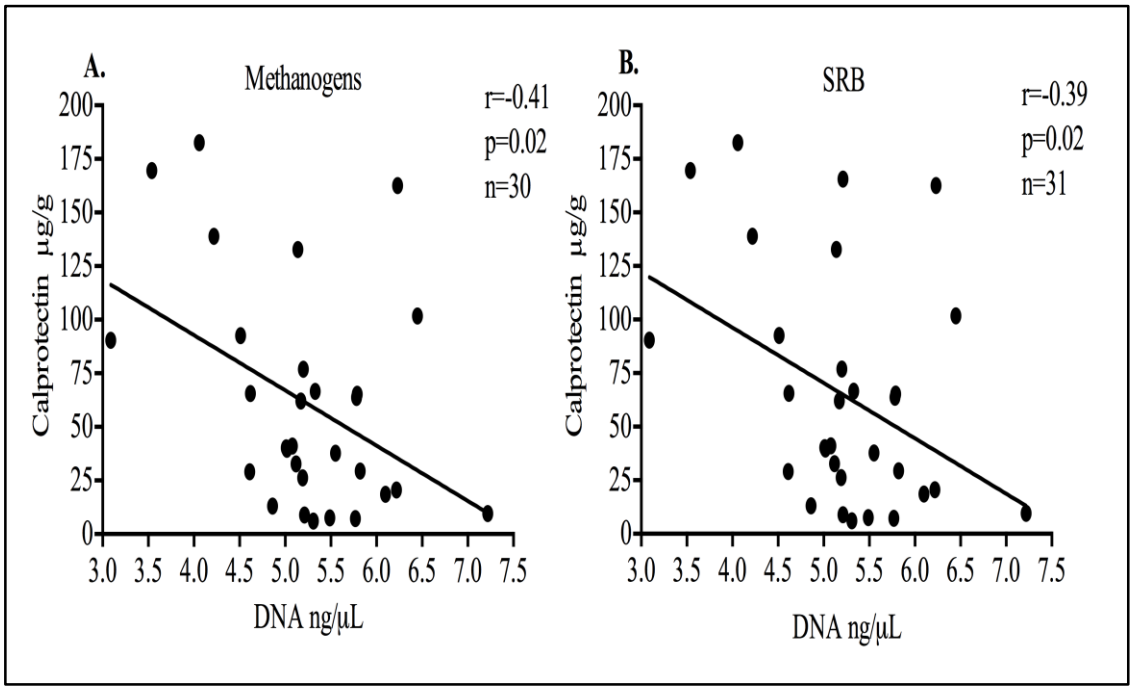

Figure 5.2. Faecal levels of colonic hydrogenotrophs and levels of inflammation at recruitment in all IBS patients. (A) Faecal Methanogen were significantly correlated with intestinal inflammation (r=-0.41 (95% CI (95% CI-0.7141 to -0.1532), p=0.02) and (**B)** SRB levels were also correlated with intestinal inflammation as measured by faecal calprotectin at recruitment (r=-0.39 (95% CI -0.6561 to -0.07005), p=0.02).

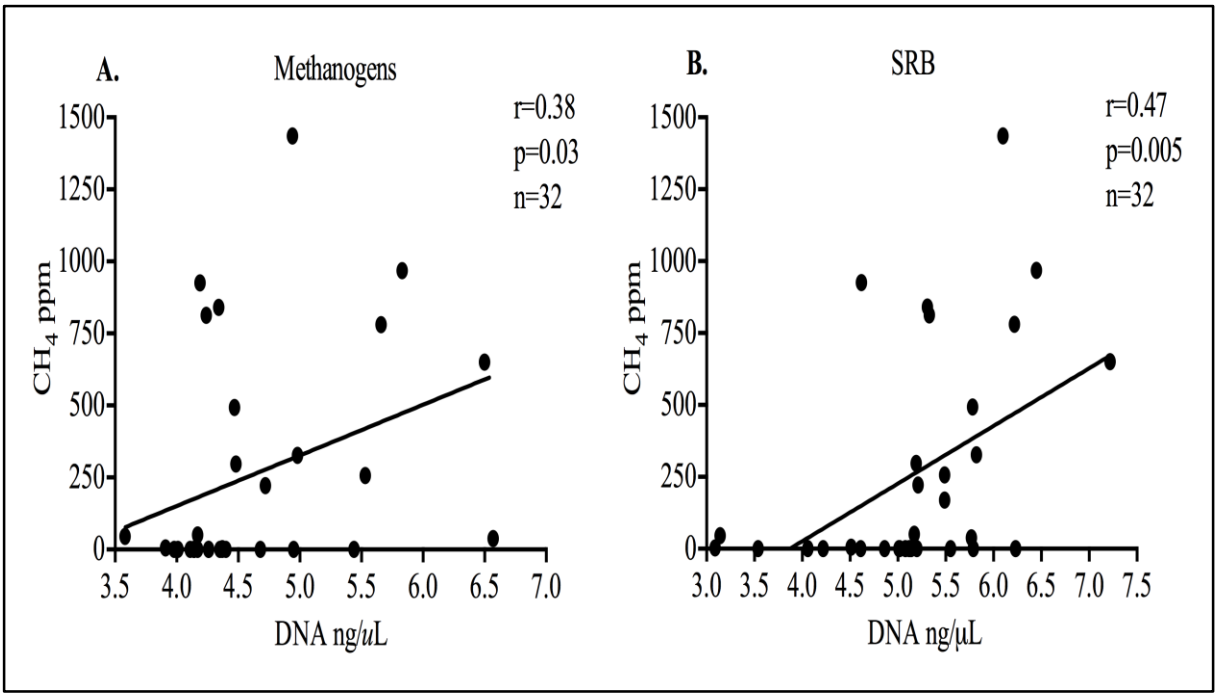

Figure 5.3. Breath CH_4 levels and levels of faecal hydrogenotrophs at recruitment. (**A**) Methanogens were significantly correlated with expired breath CH_4 (r=-0.38 (95% CI 0.0250 to 0.6571), p=0.03) and (**B**) Breath CH_4 levels were significantly correlated with faecal levels of SRB (r=0.47 (95% CI 0.04401 to 0.7843), p=0.005).

5.5.2 Section B) IBS patients according to the detection of methane in expired breath

The cohort was separated into gas type, i.e. CH_4 excretors (IBS-D n=7, IBS-C n=6, IBS-A n=4) (n=17) and non-CH_4 excretors (IBS-D n=11, IBS-C n=2, IBS-A n=1) (n=14). Significant correlations were observed between intestinal hydrogenotrophs, non-invasive intestinal indices of mucosal stress and gas production (CH_4 and H_2) over 180 minutes in CH_4 excretors (Table 5.3). There was a strong correlation between faecal intestinal hydrogenotrophs in CH_4 excretors (r=0.74 (95% CI 0.4078 to 0.0916), p=0.0006). Breath CH_4 levels were correlated with faecal levels of SRB in CH_4 excretors (r=0.52 (95% CI 0.0580 to 0.8025), p=0.03) (Table 5.3). H_2 levels were (87.06 ± 33.60) (mean ± SEM) in CH_4 excretors (Table 5.4), and were significantly lower than breath H_2 levels in non-CH_4 excretors (142.43 ± 30.94) (p=0.03) (Figure 5.5).

Table 5-3: Correlational matrix of intestinal indices, Intestinal gases and faecal hydrogenotrophs in CH_4 excretors. SRB, sulphate reducing bacteria; H_2, hydrogen; CH_4, methane; FC, faecal calprotectin (µg/g); L/R, lactulose/rhamnose ratio, (n=17), H_2 and CH_4 were measured in breath over 180 mins and expressed as ppm.

	Faecal SRB	Faecal methanogens	Breath H_2 (ppm)	Breath CH_4 (ppm)	L/R ratio	FC
Faecal SRB	1					
Faecal methanogens	0.74	1				
Breath H_2 (ppm)	-0.10	0.04	1			
Breath CH_4 (ppm)	0.52	0.18	-0.33	1		
L/R ratio	-0.33	-0.13	0.18	-0.29	1	
FC	-0.44	-0.45	-0.30	-0.21	0.07	1

Table 5-4: Expired breath CH_4 and H_2 levels at 180 minutes in CH_4 excretors, and non-CH_4 excretors. Combined levels were measured in expired breath (ppm) and are shown as mean ± SEM, H_2, hydrogen; CH_4, methane.

IBS gas type	Number in group	Breath CH_4 (ppm)	Breath H_2 (ppm)
CH_4 excretors	n=16	479.47 ± 103.5	87.06 ± 33.60
Non-CH_4 excretors	n=14	0	142.43 ± 30.94

Significant correlations were observed between intestinal hydrogenotrophs, non-invasive intestinal indices and gas production (H_2) over 180 minutes in non-CH_4 excretors (Table 5.5). Faecal SRB levels were positively correlated with breath CH_4 levels in CH_4 excretors (r=0.50 (95% CI 0.04401 to 0.7843), p=0.03) (Table 5.5). H_2 levels detected in breath were significantly correlated with small intestinal patency (r=-0.75 (95% CI -0.9309 to -0.2724), p=0.008) (Figure 5.4). L/R ratio levels were not significantly different between CH_4 excretors and non-CH_4 excretors (Figure 5.4). Faecal calprotectin levels were significantly lower in CH_4 excretors (42.30 ± 8.60) compared with non-CH_4 excretors (83.01 ± 15.11) (p=0.03) (Figure 5.4). Breath H_2 levels were significantly different in CH_4 excretors (n=17) compared with non-CH_4 excretors (n=14) (p=0.03) (Figure 5.4).

Table 5-5: Correlational matrix of intestinal indices, intestinal gases and faecal hydrogenotrophs in non-CH_4 excretors. SRB, sulphate reducing bacteria; H_2, hydrogen; FC, faecal calprotectin (µg/g); L/R, lactulose/rhamnose ratio. H_2 and CH_4 are measured in ppm, (n=14), Breath H_2 levels were measured in breath over 180 mins and expressed as ppm.

	Faecal SRB	Faecal methanogens	Breath H_2 (ppm)	L/R ratio	FC
Faecal SRB	1				
Faecal methanogens	0.50	1			
Breath H_2 (ppm)	-0.16	-0.13	1		
L/R ratio	-0.24	-0.38	-0.75	1	
FC	-0.24	-0.32	-0.16	0.24	1

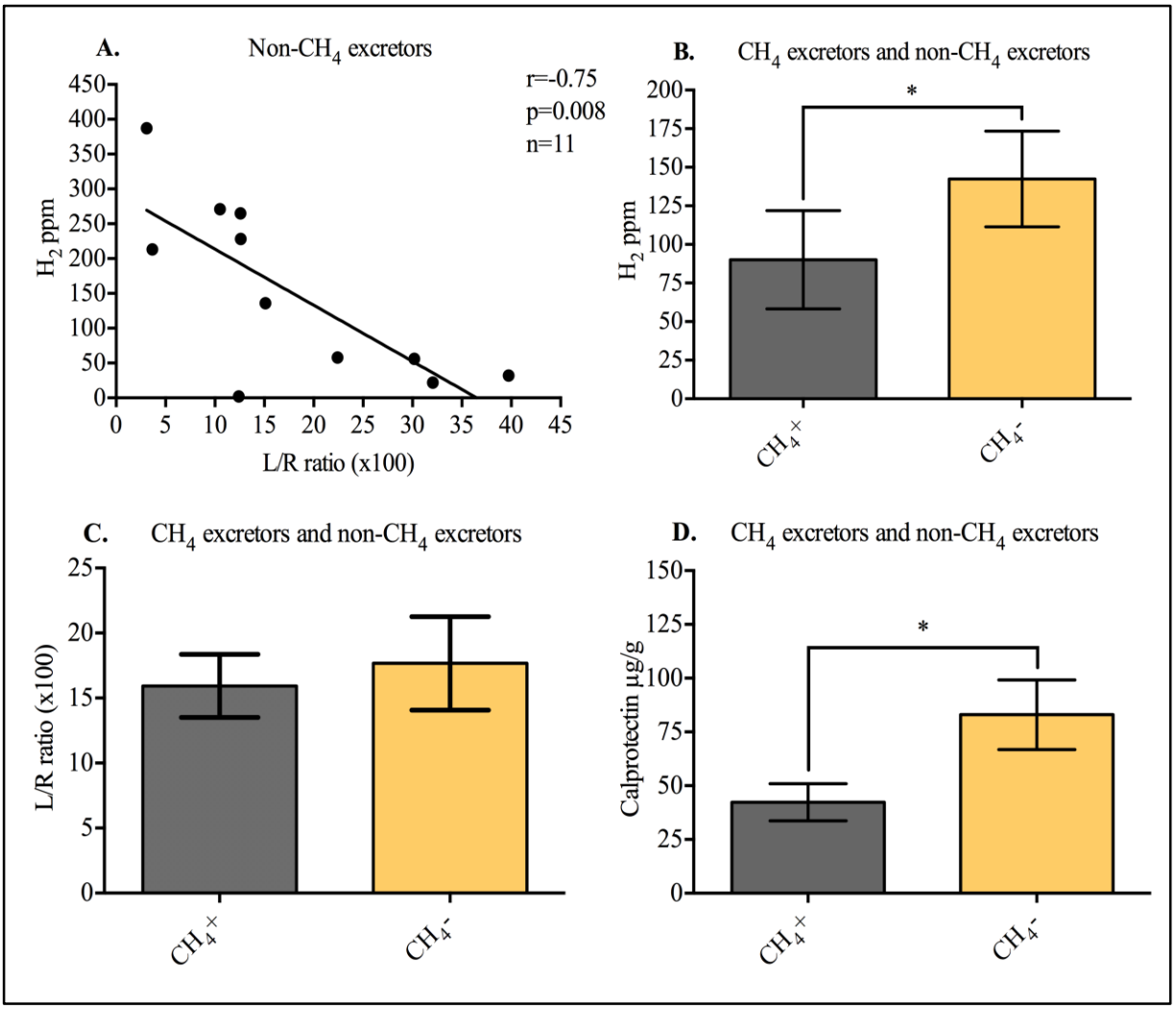

Figure 5.4. Breath H_2 levels and CH_4 levels related to indices of mucosal stress in CH_4 excretors and non-excretors. (**A**) L/R ratio is correlated with levels of H_2 in non-CH_4 excreting patients (r=-0.75 (95% CI -0.9309 to -0.2724), p=0.008), (**B**) H_2 levels were significantly lower in CH_4 excretors (n=16) compared to non-CH_4 excretors (n=14), (**C**) L/R ratio was not significantly different between CH_4 excretors and non-CH_4 excretors, (**D**) Faecal calprotectin levels were significantly lower in CH_4 excretors (42.30 ± 8.60) (n=16) compared with non-excretors (83.01 ± 15.11) (n=14). L/R, lactulose/rhamnose ratio; H_2, hydrogen; CH_4, methane; ppm, parts per million; CH_4+, CH_4 excretors, CH_4-, non-CH_4 excretors, data shown as ± SEM, *p<0.05.

5.5.3 Section C) IBS categories (IBS-D, IBS-C and IBS-A).

Section C shows the cohort separated according to IBS classification (IBS-D, n=20, IBS-C, n=8, IBS-A, n=6) (Table 5.6). There was a significant difference in breath CH_4 levels between all three phenotypes (p=0.02) (Figure 5.5). Breath CH_4 levels in IBS-D patients were significantly different compared with breath CH_4 levels in IBS-C and IBS-A phenotypes (p=0.02 and p=0.01 respectively) (Figure 5.5). Faecal methanogen and SRB levels were not significantly different among the phenotypes (Figure 5.5).

Table 5-6: CH_4 and H_2 gas detected in breath each IBS phenotype. H_2 and CH_4 levels recorded over 180 minutes, H_2, hydrogen; CH_4, methane; IBS–D, diarrhoea predominant; IBS–C, constipation predominant; IBS-A, Alternating diarrhoea and constipation. CH_4 and H_2 was measured in expired breath over 180 mins and expressed as ppm. Data shown as mean ± SEM.

IBS subtype	Number in group	Breath CH_4 (ppm)	Breath H_2 (ppm)
IBS-D	n=20	92.89 ± 47.97	120.67 ± 32.70
IBS-C	n=8	509.50 ± 198.52	128.00 ± 48.78
IBS-A	n=6	480.60 ± 161.37	55.60 ± 35.40

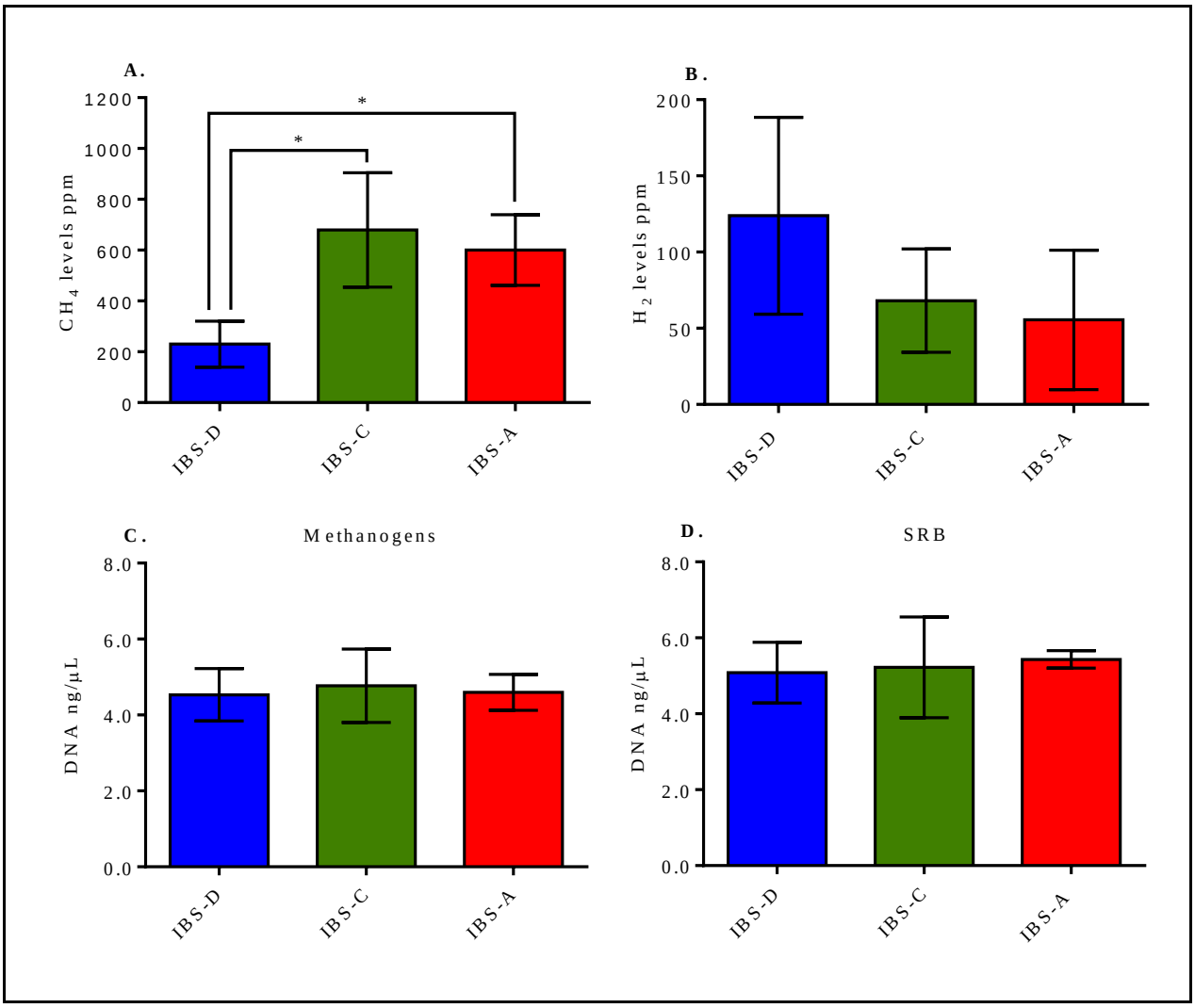

Figure 5.5. Methanogen, SRB levels and CH_4, and H_2 levels measured in breath in all IBS phenotypes. (**A**) CH_4 levels were significantly higher in both constipated and alternating IBS phenotypes compared to levels in IBS-D. (**B**) H_2 levels were not different between IBS phenotypes. (**C**) Methanogen levels were not different among IBS phenotypes and (**D**) SRB levels were not significantly different in all IBS phenotypes. IBS-D, diarrhoea (n=20); IBS–C, constipation (n=8); IBS–A, alternating (n=6); ppm, parts per million; Data shown as ± SEM, *p<0.05.

Significant correlations were observed between faecal hydrogenotrophs and non-invasive markers of mucosal stress in IBS-D patients (Table 5.7). Faecal methanogen levels were positively correlated with SRB levels in IBS-D (r=0.61 (95% CI 0.2258 to 0.8363), p=0.004) (Figure 5.6). Small intestinal patency was correlated with SRB levels in stool (r=-0.60 (95% CI -0.8455 to -0.1514), p=0.01) (Figure 5.6). Small intestinal permeability was correlated with faecal calprotectin levels in individuals with IBS-D (r=0.56 (95% CI 0.09172 to 0.8273), (p=0.02) (Figure 5.6). Faecal methanogens were weakly correlated (not significant) with the L/R ratio and faecal calprotectin levels in IBS-D patients (Table 5.7).

Table 5-7: Correlational matrix of faecal hydrogenotrophic microorganism DNA levels, intestinal gases and intestinal parameters in IBS-D. SRB, sulphate reducing bacteria; H_2, hydrogen; CH_4, methane; FC, faecal calprotectin (µg/g); L/R, lactulose/rhamnose ratio, (n=20), H_2 and CH_4 levels were measured over 180 mins and expressed as ppm.

	Faecal SRB	Faecal methanogens	Breath H_2 (ppm)	Breath CH_4 (ppm)	L/R ratio	FC
Faecal SRB	1					
Faecal methanogens	0.61	1				
Breath H_2 (ppm)	-0.19	-0.14	1			
Breath CH_4 (ppm)	0.32	-0.52	-0.32	1		
L/R ratio	-0.60	-0.39	-0.19	-0.03	1	
FC	-0.33	-0.40	-0.31	-0.52	0.56	1

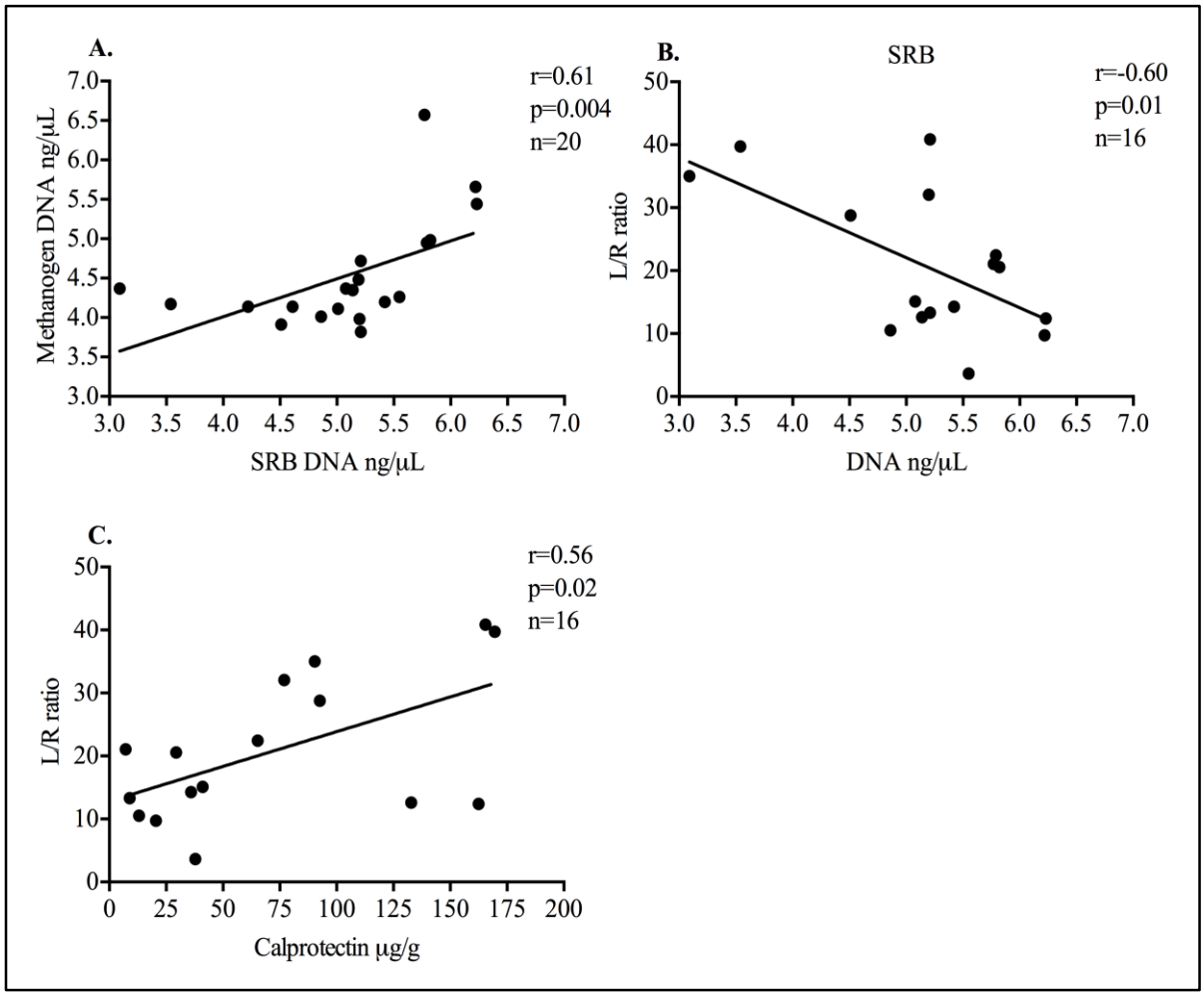

Figure 5.6. Intestinal indices and microorganisms controlling the H_2 economy in IBS-D patients. (A) Both hydrogenotrophs were positively correlated with each other in IBS-D patients (r=0.61 (95% CI 0.2258 to 0.8363), p=0.004), (B) SRB levels were correlated with a marker of small intestinal permeability (r=-0.60 (95% CI -0.8455 to -0.1514), p=0.01) and (C) L/R ratio, a marker of small intestinal permeability correlated with a marker of intestinal inflammation in IBS-D patients (r=0.56 (95% CI 0.09172 to 0.8273), p=0.02).

Positive correlations were observed between faecal hydrogenotrophs, non-invasive markers of intestinal health and intestinal gases measured in breath in IBS-C patients (Table 5.8). There was a significant positive relationship between faecal SRB and methanogen levels in IBS-C patients (r=0.93 (95% CI 0.6646 to 0.9880), p=0.0007) (Figure 5.7). CH_4 and H_2 levels in breath were highly correlated in IBS-C patients (r=-0.70 (95% CI -0.9400 to 0.01583), p=0.05) (Figure 5.7).

Table 5-8: Correlational matrix of faecal hydrogenotrophic microorganism DNA levels, intestinal gases and intestinal parameters in IBS-C patients. SRB, sulphate reducing bacteria; H_2, hydrogen; CH_4, methane; FC, faecal calprotectin (µg/g); L/R, lactulose/rhamnose ratio. (n=8), H_2 and CH_4 were measured in breath over 180 mins.

	Faecal SRB	Faecal methanogen	Breath H_2 (ppm)	Breath CH_4 (ppm)	L/R ratio	FC
Faecal SRB	1					
Faecal methanogens	0.93	1				
Breath H_2 (ppm)	-0.47	-0.28	1			
Breath CH_4 (ppm)	0.60	0.46	-0.70	1		
L/R ratio	-0.13	-0.28	-0.13	-0.09	1	
FC	-0.40	-0.40	-0.20	-0.40	0.51	1

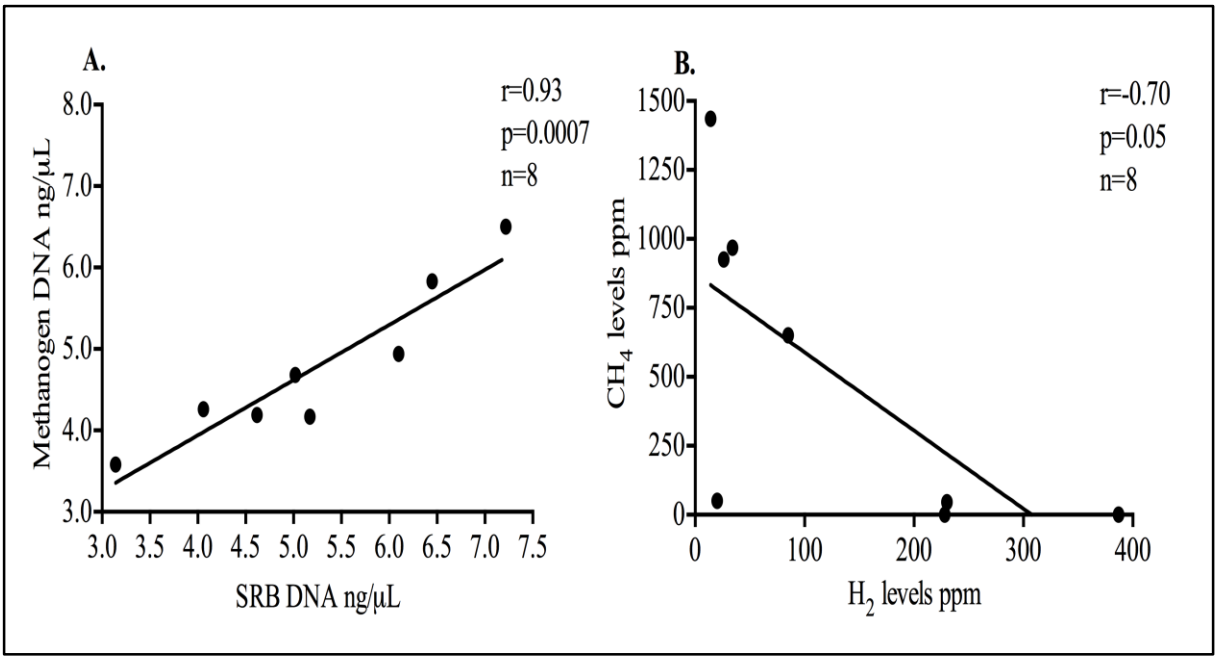

Figure 5.7. Hydrogenotrophic microorganisms controlling the H_2 economy in IBS-C patients. (**A**) Faecal methanogen levels were significantly correlated with faecal levels of SRB in IBS-C patients (r=0.93 (95% CI 0.6646 to 0.9880), p=0.0007) and (**B**) Expired breath CH_4 levels were correlated with breath H_2 levels in IBS-C (r=-0.70 (95% CI -0.9400 to 0.01583), p=0.05).

5.6 Discussion

This study quantitatively analysed selected microorganisms associated with determining the H_2 economy in the colon and compared them with non-invasive markers of a stressed mucosa, and H_2 and CH_4 levels measured in expired breath in a cohort of IBS patients. Potentially important relationships were observed between indicators of mucosal stress, non-invasive measurements of microbial fermentative activity (breath H_2 and CH_4) and faecal levels of selected hydrogenotrophic microorganisms in the GIT. One of the most important observations from this study was that higher levels of breath H_2 were significantly associated with increased tightening of the mucosal barrier as measured by the L/R ratio in non-CH_4 excretors. In addition, in CH_4 excretors a significantly lower faecal calprotectin level was seen compared to non-CH_4 excretors.

The H_2 economy has received relatively little attention, considering that hydrogenotrophic microbes play such an essential role in digestive biochemistry. Moreover, relatively little is known of the potential of intestinal gases governed by hydrogenotrophs and their role as markers of gastrointestinal health. The interesting associations reported in this study suggest that higher levels of H_2 in breath occurred with increased tightening of the junctions between enterocytes lining the small intestine, measured by the L/R ratio in IBS-D patients. A possible explanation of this phenomenon is that H_2 produced through fermentation acts as a local antioxidant preventing oxidative damage at the mucosal level. Recent studies have shown that H_2 has antioxidant properties in various animal models and may be used therapeutically. In rats, 2% H_2 inhalation preoperatively prevented post-surgical transplantation-induced intestinal injury by decreasing intestinal permeability and reducing inflammatory mediators

such as TNF-α (Buchholz, Kaczorowski, Sugimoto *et al*. 2008). H_2 inhalation decreased histological markers of mucosal damage such as villus height, compared to animals not receiving H_2 (Buchholz, Kaczorowski, Sugimoto *et al*. 2008). In another study using a dextran sodium sulphate (DSS) model of IBD in mice, Kawai *et al* 2009, reported that mice given DSS plus 5% solution of H_2 in water had significantly reduced clinical symptoms of DSS induced colitis compared with mice receiving DSS alone. (Kajiya, Silva, Sato *et al*. 2009). F4/80 positive macrophages are reported to increase during mucosal damage caused by DSS induced colitis (Kajiya, Silva, Sato *et al*. 2009). Mice receiving DSS + H_2 showed significantly decreased levels of F4/80 macrophage infiltration into the colonic mucosa indicating that H_2 reduced mucosal migration (Kajiya, Silva, Sato *et al*. 2009).

In another study using concanavalin-induced hepatitis in mice, H_2 produced by intestinal bacteria decreased the amount of oxidative damage caused by this mitogen. Concanavalin induces a massive inflammation and necrosis of hepatocytes and is used to mimic the properties of autoimmune hepatitis (Kajiya, Sato, Silva *et al.* 2009). Mice that received antibiotics had a significantly altered microbiota (decreased density) and significantly more hepatocyte damage than mice not administered antibiotics after concanavalin administration. Furthermore, H_2 levels were measured in the caecum, small intestine, large intestine, spleen and liver of mice without antibiotic treatment, and were increased compared to mice treated with antibiotics. This confirmed that levels of H_2 in these organs was directly related to the H_2 produced by the intestinal microbiota (Kajiya, Sato, Silva *et al*. 2009). The authors also reported that increased levels of H_2 suppressed the pro-inflammatory cytokines TNF-α and

interferon gamma (IFN-γ), suggesting that circulating H_2 may play a role in mediating inflammation in this model (Kajiya, Sato, Silva *et al.* 2009).

When the IBS patients in the current study were separated into gas type (CH_4 excretors and non-CH_4 excretors), there was a significant correlation between H_2 levels and increased small intestinal patency in non-CH_4 excretors. Conversely, CH_4 excretors, as expected, had significantly reduced H_2 levels with no relationship seen between the level of H_2 and small intestinal permeability. This supports the suggestion that a colonic fermentation profile producing a certain level of H_2 can act as an antioxidant to reduce mucosal stress in the small intestine. Furthermore, H_2 production, a proportion of which subsequently is absorbed into the systemic circulation and bathes other organs, may be beneficial in other organs. Thus strategies for the management of the H_2 economy may have important implications for mucosal health, and more broadly for homeostasis of other organs. Prospective well-designed studies using larger numbers are needed to test this hypothesis.

Another interesting finding of this study was that the levels of intestinal inflammation were significantly lower in CH_4 excretors compared to non-CH_4 excretors, suggesting that CH_4 production may be associated with a less inflamed mucosa. It may be that CH_4 levels are signalling a recovery from diarrhoea with a concomitant slowing of gut motility. The well-documented higher proportion of CH_4 excretion in patients with constipation (Soares, Lederman, Fagundes-Neto *et al.* 2005, Chatterjee, Park, Low *et al.* 2007) would be in agreement with this. Indeed, a number of studies have found that expired CH_4 is absent or greatly reduced in patients with the severe inflammatory conditions of UC and CD compared

to controls (Bjørneklett and Jenssen 1982, Mckay, Eastwood and Brydon 1985). In other studies, breath CH_4 was only detected in 2 out of 78 subjects with IBD in this dominantly diarrhoeal and highly inflammatory condition (Pimentel, Mayer, Park *et al.* 2003). This data also suggests that fermentative patterns combined with non-invasive indicators of mucosal stress may have diagnostic value in subsets of IBS patients. The categorization of IBS may be improved by incorporating the fermentative status, i.e. CH_4 excretors or non-CH_4 excretors and levels of H_2 production, into the diagnostic criteria as it appears to be altered in different classifications of IBS. Indeed, altered fermentative activity has been reported in IBS patients compared to controls (King, Elia and Hunter 1998).

There is little available information regarding the functional dynamics of methanogenic archaea and CH_4, and their role in intestinal health. Recently, levels of faecal methanogenic archaea were reported to be higher in faecal samples from patients with polyps, and patients with colorectal adenocarcinoma compared with controls, indicating that archaea may be a possible marker of GIT health (Mira-Pascual, Cabrera-Rubio, Ocon *et al.* 2014). Others have reported increased levels of methanogenic archaea in patients with diverticulosis (Weaver, Krause, Miller *et al.* 1986). Results from the current study show an association with decreased intestinal motility and breath CH_4, which is consistent with other reports in the literature (Soares, Lederman, Fagundes-Neto *et al.* 2005, Chatterjee, Park, Low *et al.* 2007, Furnari, Savarino, Bruzzone *et al*. 2012). Interestingly, in this study faecal levels of methanogens did not differ significantly among the different IBS phenotypes. This is an interesting finding as it suggests that the activity of methanogens (determined by an increase or decrease in breath

CH_4) may be influenced more by environmental conditions specific for each classification of IBS. Further studies are needed to investigate this proposition.

This cohort of IBS patients was unusual in that <10% had evidence of SBBO (Table 5.1). Others have reported prevalence of SBBO ranging from 30% to 80% (Pimentel, Chow and Lin 2000, Nucera, Gabrielli, Lupascu *et al.* 2005, Grover, Kanazawa, Palsson *et al.* 2008) in IBS patients. Patients with SBBO show early H_2 production after administration of lactulose (a synthetic sugar not digested by humans), indicating the inappropriate colonization of bacteria in the small intestine (Bryant, Fraser, Vozzo *et al.* 2004, Tooley, Howarth and Butler 2009). Treatment of patients with SBBO with the non-absorbable antibiotic Rifamixin have shown decreased transit times (alleviation of diarrhoea) in patients where diarrhoea is the dominant symptom. The authors of this study suggested that the increased H_2 production (via abnormal fermentation in the small intestine) may induce a hypercontractile state (Majewski, Reddymasu, Sostarich *et al*. 2007). The patients in the present study produced similar levels of breath H_2 but this was derived predominantly from colonic fermentation, as 31 of the 34 patients showed no SBBO using the lactulose breath test.

Another finding from this study was the strong significant correlations between methanogens and SRB and between SRB levels and breath levels of CH_4 in CH_4 excretors. These findings are interesting in light of others work that SRB and methanogens are mutually exclusive. One study using culture-based techniques of faecal slurries found that increased levels of SRB were associated with significantly reduced levels of methanogens and breath CH_4 (Gibson, Macfarlane and Macfarlane 1993). This was in a healthy cohort and used culture-based

methods that may not capture all bacteria present, as the newer molecular techniques do. Furthermore, it may not be reflective of IBS patients. Although sulphate reduction was not directly measured in this study the increased levels of SRB could indicate increased production of H_2S, which may irritate the mucosa. The association between these two hydrogenotrophs may be a marker of dysbiosis, as they appear to be mutually exclusive in studies with healthy volunteers (Gibson, Cummings and Macfarlane 1988, Gibson, Macfarlane and Cummings 1988, El Oufir, Flourie, Des Varannes *et al.* 1996). Indeed, Strocchi *et al* (1994), found that methanogens outcompeted SRB for H_2 in the faecal samples from healthy human donors, illustrating that methanogens were more efficient at utilizing H_2 than SRB (Strocchi, Furne, Ellis *et al.* 1994).

In other studies, Bryant *et al* (1977), reported that SRBs and methanogens may have a syntrophic relationship via interspecies H_2 transfer in culture (Bryant, Campbell, Reddy *et al*. 1977), but this is yet to be established in the human GIT (Nakamura, Lin, Mcsweeney *et al.* 2010). This syntrophy may explain the correlation between these two hydrogenotrophs in this IBS cohort. In a sulphate depleted methanogenic bioreactor large numbers of SRB were reported, indicating that SRB were able to grow under altered environmental conditions (Raskin, Rittmann and Stahl 1996), which may also help to explain the strong correlation between SRB and methanogens observed in the current study. *In vivo* it is unclear what the interrelationship between SRB and methanogens is. However it may be that the intraluminal environment in IBS is dominated by host-microbiome interactions. This remains to be investigated incorporating non-invasive biomarkers of host response and molecular

techniques for assessing both microbiome composition and activity in larger randomized clinical trials of IBS.

A recent study reported that reducing H_2S production in healthy rats resulted in increased mortality and increased mucosal injury in both the small intestine and colon in a trinitrobenzene sulfonic acid (TNBS) model of colitis in rats (Wallace, Vong, Mcknight *et al.* 2009). After intra-colonic administration of TNBS rats were administered intra-colonic H_2S and killed at various times post treatment. H_2S administration reduced mucosal injury and decreased expression of TNF-α. This study suggested that H_2S production might modulate certain inflammatory pathways in colitis. In this current study in IBS patients, where inflammation is of a much lower grade, SRB levels were inversely correlated with faecal calprotectin levels, and although this study did not measure colonic H_2S levels it suggests that a certain level of SRB may modulate intestinal inflammation. There was also an association between faecal levels of SRB and small intestinal permeability in the IBS-D cohort. This may suggest that certain levels of SRB are beneficial for the mucosa, as they appear to influence small intestinal patency in this subset of IBS patients. It is important to note that Muyzer and Stams (2008), have stated that high levels of SRB or SRB occurrence may not necessarily correlate with H_2S production (Muyzer and Stams 2008). Moreover, whether or not SRB concentrations, or by extension H_2S production or other metabolites produced by SRB, influence small intestinal permeability is as yet unknown. In a recently published study, H_2S was found to influence regulatory T cell (Treg) differentiation and function via fork head family transcription factor (Foxp3) (Yang, Qu, Zhou *et al.* 2015). In H_2S deficient mice Treg cells were diminished in mouse spleen and lymph nodes leading to

autoimmune disease. This study showed that H_2S was needed to sulfhydrate specific transcription factors that led to the recruitment and maintenance of Foxp3 demethylation, which promoted immune system homeostasis (Yang, Qu, Zhou *et al.* 2015). Presently, there is little information regarding the correct proportion of SRB within the intestinal microbiome and their metabolic activity in regards to health. The data from this current study suggests that SRB may have a role in decreasing inflammation in the GIT.

5.7 Conclusions

This study provides the first quantitative assessment of microorganisms associated with the H_2 economy and non-invasive measures of mucosal stress and bacterial fermentation activity in a cohort of IBS patients. It highlights the potential for the combined use of fermentative gases measured in breath, parameters of tight junction integrity and specific faecal microorganisms as non-invasive biomarkers of a stressed mucosa. In terms of fermentative activity of the microbiome, more attention has been directed at analysing metabolites such as SCFAs, with less emphasis on the state of the fermentative compartment as determined by breath H_2 and CH_4. Moreover, the excretion of H_2 and CH_4 in breath has received little attention in relation to parameters of mucosal stress. This study has shown associations with specific non-invasive markers of mucosal stress and intestinal gases related to the H_2 economy in IBS patients, with these potentially useful as functional markers of a stressed mucosa. These associations need to be investigated in future clinical studies with the use of non-invasive biomarkers to aid in diagnosis and to establish mechanisms to monitor efficacy of proposed interventions in subsets of IBS patients.

Chapter 6.0 Summary and conclusions

6.1 Introduction

Mucosal stress is a growing field of interest with anecdotal and emerging empirical evidence supportive of compositional shifts in the microbiome and disturbed barrier function prior to a number of syndromes and illnesses (Bischoff, Barbara, Buurman *et al.* 2014). This thesis has focussed on time course events in acute episodes of diarrhoea in the most damaged setting, through to the recovery phase of chemotherapy induced mucosal damage. It has contrasted this with acute virally induced diarrhoea in infants with a known and unknown mechanism of action, and a chronic low-grade inflammatory illness of unknown aetiology.

The most important findings of this thesis are the observations that the composition and activity of the microbiome differs in diseases and syndromes characterized by mucosal damage and mucosal stress. Where non-invasive biomarkers of a stressed or inflamed mucosa have been able to be measured, relationships between the endogenous microbial composition and activity may significantly influence host response, severity, progression and duration of disease. Relatively few studies have specifically investigated the balance of the faecal microbiome and the fermentation activity of the colon and its association with non-invasive biomarkers of mucosal dysfunction in gastrointestinal diseases of differing severity.

6.2 Mucosal stress induced by chemotherapy

Chemotherapy-induced mucositis affects the entire alimentary tract from mouth to anus and is an outcome of chemotherapy treatment for a range of cancers. The severe diarrhoea observed in patients receiving chemotherapy highlights the severity of mucosal stress and the need for effective treatments. Studies in human patients with cancer undergoing chemotherapy preclude invasive studies including endoscopic biopsy. In Chapter 2, using a tumour bearing animal model, mucosally protective endogenous beneficial *Lactobacillus* and *Bifidobacterium* spp. were drastically reduced during irinotecan treatment, with a rebound to pre-irinotecan levels when treatment was ceased. This pattern also reflected the occurrence of mucosal damage induced by irinotecan administration and the healing cycle once treatment had ceased. The dramatic reduction of the endogenously beneficial microorganisms coincided with the significant elevation of the potentially pathogenic *E. coli.* In this model the β-glucuronidation activity of *E. coli* increased the toxicity of the chemotherapeutic agent irinotecan by metabolizing the excreted glucuronidated compounds to its active metabolite (SN-38). This mechanism increases the severity and duration of mucosal damage (Stringer, Gibson, Logan *et al.* 2008). Indeed, the markedly increased ratio of *E. coli* to beneficial microbes provides a niche for *E. coli* to dominate the luminal and mucosal environment in both the caecum and colon further compounding the level of mucosal damage and stress. Observing the compositional shifts in faeces; before during and after chemotherapy, in conjunction with functional tests to evaluate the gastrointestinal mucosa might potentially be useful in defining patients more vulnerable to treatment side effects. Moreover, if the ratio of potentially harmful microbes to beneficial microbes does play a causative or additive role in mucositis, which has yet to be conclusively shown, then

understanding the composition of the endogenous microbiome before treatment may help separate good responders from poor responders.

Understanding the composition of the endogenous microbiome pre-treatment, may allow for selective augmentation of beneficial microorganisms in patients with low endogenous levels. Evidenced based interventions, using a range of prebiotics/probiotics and synbiotics, based on the observed faecal composition may be investigated in the future. In a recently published human trial, patients receiving a range of different chemotherapeutic agents were shown to have decreased levels of beneficial bacteria such as *Bifidobacterium* spp., *F. prausnitzii,* and *Rosburia* spp. This was shown in faeces and was associated with increased intestinal permeability post treatment (Montassier, Gastinne, Vangay *et al.* 2015). Increased permeability and inflammation was attributed to the decreased levels of the aforementioned protective bacteria to dampen inflammatory pathways including; TNF-α, NFκB and lipopolysaccharide inflammatory responses (Montassier, Gastinne, Vangay *et al.* 2015). The ability to actively alter the levels of these beneficial microorganisms while decreasing those that may be deleterious presents an exciting range of therapeutic options.

In this thesis caecal and colonic contents were collected at each time point to analyse the regional variations of the microbiota in these two gastrointestinal compartments after irinotecan administration. Rats are caecal fermenters with the caecum seeding the colon. Faeces have been predominately used in human studies of the microbiome, as they are non-invasive and relatively easy to collect. This current study did not assess faecal levels but rather focussed on the collection of luminal samples in direct contact with the damaged

mucosa. It is still to be determined how accurately the faecal levels reflect the microorganisms in the various compartments of the GIT (Zoetendal, Von Wright, Vilpponen-Salmela *et al.* 2002). Colonic contents more so than caecal contents likely reflect levels of microorganisms in the entire GIT recovered in the faeces, but this has not been definitively and systematically confirmed. Future studies should look at these relationships. The current study had a small sample size and faecal levels were not performed. Nonetheless, the time course associations are clear and the ratios correlate well with the damage suggesting that faecal ratios may provide a useful non-invasive diagnostic surrogate of the degree of mucosal stress (Stringer, Gibson, Bowen *et al.* 2009).

6.3 Acute viral gastroenteritis and mucosal stress in children

In a paediatric cohort with RV and the newly described HBoV2 there was a significant association with increased levels of beneficial endogenous *Bifidobacterium* spp. and decreased bowel movements in RV infection compared to healthy controls. This may indicate that the endogenous *Bifidobacterium* spp. protected the mucosa from the harmful toxin NSP4 responsible for the osmotic diarrhoea observed during RV infection. Another possible explanation for the correlation with decreased bowel movements is the production of SCFAs, as these have been shown to decrease water loss in the colon, which could possibly decrease diarrhoea (Binder 2010). *Bifidobacterium* spp., from human and bovine origin, and specifically an exopolysaccharide produced by them, has been shown to beneficially alter the composition of the certain bacteria within the microbiome leading to the increased production of SCFAs (Salazar, Ruas-Madiedo, Kolida *et al.* 2009).

Changes were also observed in the microbiome in both viral infections when compared to healthy controls. The endogenous beneficial bacteria *Bifidobacterium* and *Lactobacillus* spp. have been arguably used for centuries as probiotics administered to maintain intestinal health, the mechanisms and degree of efficacy however are still poorly understood (Boirivant and Strober 2007). There is even less information regarding the endogenously occurring beneficial bacteria and their role in protecting the mucosa under stress (Bourlioux, Koletzko, Guarner *et al.* 2003). Additionally, there is a paucity of information on the long-term implications for mucosal recovery after gastrointestinal infections. Little is known of the long-term impact on the compositional stability of the microbiome once the viral illness has resolved and how this may affect health (Thabane, Simunovic, Akhtar-Danesh *et al.* 2010). There is evidence to suggest, as is the case with post infectious IBS, that both the intestinal microbiome and the intestinal mucosa may not fully recover after acute gastroenteritis caused by a bacterial pathogen (Thabane, Simunovic, Akhtar-Danesh *et al.* 2010). Whether or not this is this case for post viral sequelae in childhood infections is currently unknown. Compositional shifts in the intestinal microbiome may predispose individuals to immune system dysregulation via increased intestinal permeability triggering inflammation (Codling, O'mahony, Shanahan *et al.* 2010). There is clearly a place for the application of non-invasive markers of mucosal stress in the recovery phase, but also as an ongoing method to monitor and detect mucosal stress post disease.

The findings in this current study are interesting in the context of exogenous probiotic use during episodes of diarrhoea caused by viral infections such as RV. The use of *Lactobacillus rhamnosus* in the treatment of RV has shown decreased duration and severity of RV infection in a number of randomised trials (Guandalini, Pensabene, Zikri *et al.* 2000, Szajewska,

Skorka, Ruszczyński *et al.* 2007). In other studies paediatric patients with IBS receiving *L. rhamnosus* have shown decreased small intestinal permeability (Francavilla, Miniello, Magistà *et al.* 2010). Conversely, in indigenous Australian children with diarrhoeal illness the administration of *L. rhamnosus* did not alter the course of RV infection or change intestinal permeability measured by the L/R ratio compared to placebo (Ritchie, Brewster, Tran *et al.* 2010). These results may be indicative of the underlying environmental enteropathy present in these children, which more than likely coincides with a more hostile microbial environment compared with children from an urban setting and a different socioeconomic demographic.

E. coli levels during both viral infections were significantly raised compared to control levels. These findings appear to suggest that the luminal and possibly the mucosal environmental conditions during different viral infections are changed leading to increased levels of *E. coli*. However, *E. coli* levels in HBoV2, where the frequency of diarrhoea appeared to be more severe, were similar compared to the RV group. *E.coli* levels alone may not discriminate between the severity of the two viral infections and this may be a consequence of the sample size. In contrast to *E. coli* the levels of *Bifidobacterium* spp. were not significantly different compared with the healthy controls. However, the levels in HBoV2 infection were significantly lower than those seen in RV infection. Measuring the beneficial bacteria alone, or assessing *E. coli* levels in faeces in isolation may not provide a useful biomarker of the status of gut health in these settings. In section 6.7 the possible use of *E. coli:Bifidobacterium* spp. (EC:BF) ratio is proposed as a potential biomarker of a stressed mucosa.

6.4 Mucosal stress and irritable bowel syndrome

The current study has reported significant associations with non-invasive markers of mucosal stress, selected microorganisms of the microbiome and the fermentative activity in IBS patients. IBS has elements of low-grade inflammation and increased intestinal permeability affecting mucosal immunity leading to a constellation of symptoms, which makes the management, and diagnosis difficult. Currently, there are no routinely accepted non-invasive biomarkers for the diagnosis of IBS. The microbiome represents not only a reservoir of potential biomarkers for the diagnosis of IBS but also a reservoir of beneficial endogenous microorganisms with therapeutic potential. Microbial indicators (such as levels or ratios of particular microorganisms), combined with information available from non-invasive breath tests and parameters of altered barrier function may provide new innovative methods of understanding IBS in the clinical setting. Recent work in IBD has suggested a role for an *E. coli:F. prausnitzii* ratio for differential diagnosis of IBD vs. IBS (Lopez-Siles, Martinez-Medina, Busquets *et al.* 2014). The studies in IBS in this thesis support this contention but also suggest possible ways to better characterise different IBS subsets from a mechanistic point of view using these biomarkers. Recent work has also shown in IBD that the viriome appears to increase its diversity in concert with a diminished diversity of the bacteriome. It is not known whether these types of changes are seen in IBS. IBS is characterised as a lower grade inflammation, and whilst this thesis did not address the microbiome diversity, it does appear that future work should focus on the shifts and the broader changes related to barrier dysfunction and severity of stresses to the mucosal niche including various grades of inflammation.

The findings from this thesis have shown that increased faecal levels of mucosally protective endogenous microorganisms were associated with a more stable mucosal environment as measured with non-invasive markers of small intestinal permeability and inflammation. Mucosal levels of microorganisms represented in faeces and their relationship to non-invasive markers of mucosal stress needs to be further evaluated in large well-designed prospective studies. Indeed, the findings from this study agree with others that have reported the beneficial attributes of *F. prausnitzii* and *A. muciniphila* and the well-studied *Lactobacillus* spp. and *Bifidobacterium* spp.

6.5 The hydrogen economy

The findings of the current study suggest that the activity of the microorganisms controlling the H_2 economy is an important factor to understand in regards to potential host responses. Certainly, this has been shown in certain classifications of IBS patients in this study (IBS-D, IBS-C and IBS-A). All patients harboured methanogens and the faecal levels observed were similar in each category. However, CH_4 was not detected in breath in all patients. Not surprisingly, non-CH_4 excretors had higher levels of H_2 and this was significantly correlated with tightening of the small intestinal mucosa. No such relationship was seen in CH_4 excretors where the H_2 levels were significantly lower. Another interesting finding from this study was that in CH_4 excretors a significantly lower level of mucosal inflammation was observed compared to non-CH_4 excretors. This data infers that CH_4 production may be a barometer of the state of mucosal health at the time of sampling. This suggests that the activity of the microbiome and the environment promoting the activity is important and may have beneficial consequences. The finding of others, and the data presented in this study are in agreement with the contention that CH_4 excretors are more likely to be constipated. The data in this study

also showed that IBS-D patients were predominantly non-CH_4 excretors and produced lower levels of CH_4 on breath than CH_4 excretors overall. Stimulating CH_4 production in IBS-D patients may be a potential therapeutic option to slow motility in this subset of IBS patients. Conversely, manipulating the microbial environment to decrease methanogenesis may potentially alleviate the symptoms in IBS-C patients. The observations in Chap 5 were seen in a low-grade mucosal inflammatory background with some indices of mucosal stress as determined by a raised small intestinal permeability. As such, this differs from Chap 2 and Chap 3 where mucosal damage was much higher. The role of methanogens and production of CH_4 and H_2 needs to be examined in these acute settings using non-invasive biomarkers to determine any relationship with mucosal damage and stress.

A keystone species is an organism within an environmental niche, which exerts a far greater effect relative to the numerical amount within that particular ecosystem (Power, Tilman, Estes *et al.* 1996). Methanogenic archaea may be thought of as one such species, as there is at present predominately only one archaea identified. This microbe, *Mbb. smithii* and a group of SRB in the human GIT maintain a reduced environment in the large bowel by performing the vital role of hydrogen gas regulation and disposal. Methanogenic archaea have been shown to outcompete SRBs for H_2 from healthy human donors using *in vitro* techniques (Figure 6.1) (Strocchi, Furne, Ellis *et al.* 1994), which supports their putative keystone status. The disposal of H_2 with the consequent production of CH_4 enables a range of bacteria to increase the fermentative activity and increase the amount of energy salvaged from the diet. *Mbb. smithii* the dominant archaeon in the human GIT shows enrichment in genes for the usage of CO_2 and H_2 (Arumugam, Raes, Pelletier *et al.* 2011). Furthermore, *Mbb. smithii* may also encode for genes involved in acetate assimilation (Arumugam, Raes, Pelletier *et al.* 2011). These

attributes appear to promote a syntrophic relationship with other microbes in the GIT. In this regard the relationship between CH_4 excretion and reduced levels of intestinal inflammation observed in this thesis needs to be investigated in large well-designed prospective studies to test this finding.

Figure 6.1. Balance of major bacterial metabolites in the luminal environment.

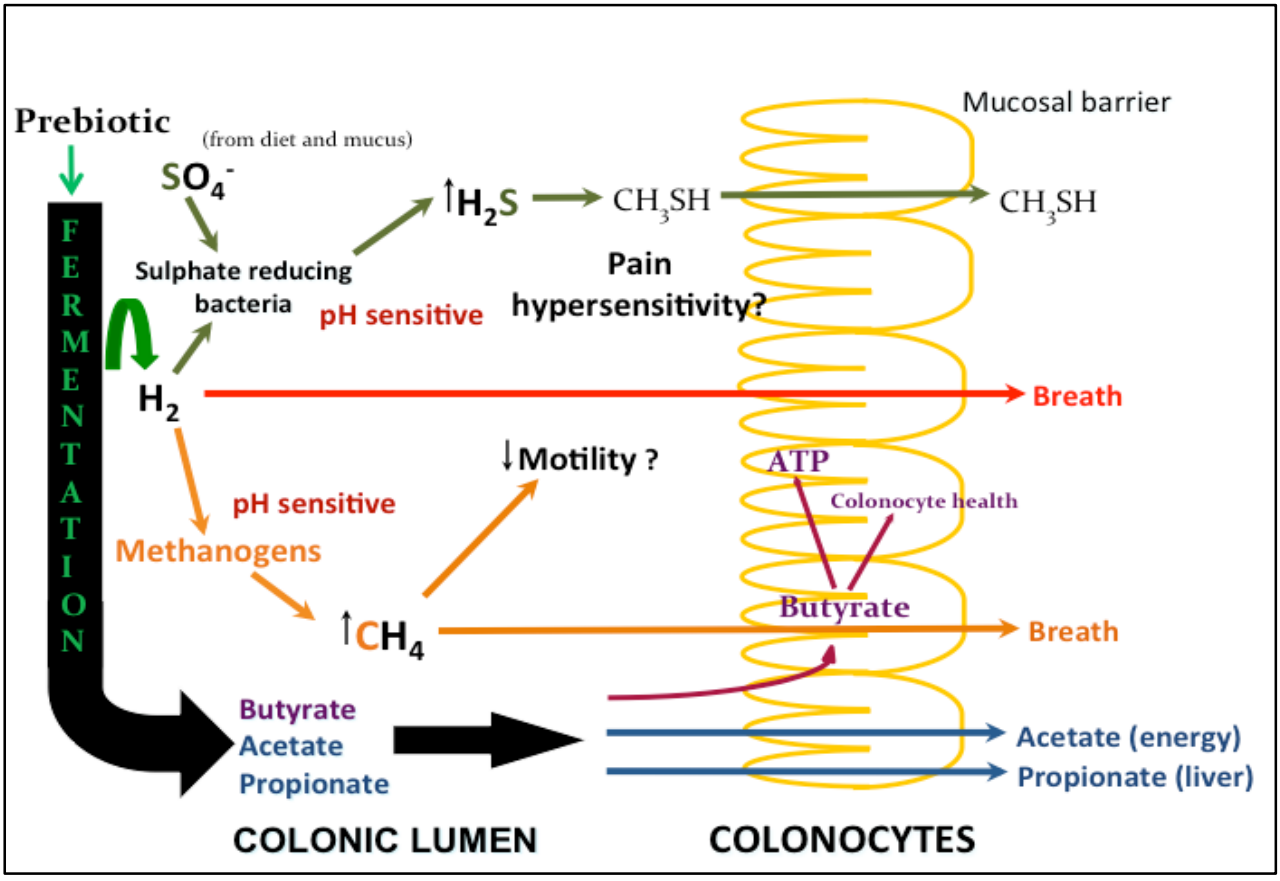

Figure 6.1. The major hydrogenotrophic microbes governing the hydrogen economy have different optimal pH levels, which may affect activity in the different geographical regions of the GIT. H_2S may irritate the mucosa and CH_4 may affect motility leading to constipation. Methanogens are thought to outcompete SRB for H_2 in the colon and thus can be regarded as a keystone species.

The question of whether *Mbb. smithii* and other archaea residing in the human GIT are potential pathogens is as yet unresolved. There have been reports of increased levels of methanogens in obese people, with the assumption that they increase colonic salvage form ingested carbohydrates leading to increased adiposity (Zhang, Dibaise, Zuccolo *et al.* 2009). There is also the finding that methanogens are raised in anorexic patients (Armougom, Henry, Vialettes *et al.* 2009), and CH_4 excretion is increased in patients with cystic fibrosis (Bujanover, Peled, Blau *et al.* 1987). This may suggest that *Mbb. smithii* stimulates other fermentative microorganisms, to extract the maximum amount of energy from the diet (as it is sporadic in the case of anorexia), or it may suggest that colonic mucins become a sole source of carbohydrate for bacteria, with degradation of this plentiful resource facilitated by *Mbb. smithii.*

Currently, oxidative stress is seen as a major issue in a growing number of diseases. Interestingly, in the IBS study levels of hydrogen gas detected in breath were correlated with increased small intestinal patency. The results from this study support recent findings showing the introduction of H_2 supported mucosal function in the DSS model of colitis (Kajiya, Silva, Sato *et al.* 2009). The microbiota of healthy rats was decreased using antibiotics and there was a direct correlation with H_2 levels detected in remote sites such as liver and lungs with the microbiota (Kajiya, Sato, Silva *et al.* 2009). Manipulation of the microbiome to either increase H_2 production, to increase intestinal patency or to change the microbiome activity of a non-CH_4 excretor to a CH_4 excretor to slow motility, may have beneficial consequences in particular settings where mucosal stress is implicated.

6.6 Non-invasive monitoring of the mucosa under stress

6.6.1 Current assessment of intestinal permeability

Presently clinicians are hindered by methods to evaluate the functional state of the intestinal mucosa in vivo under normal and perturbed settings in both human and animal models. Highly invasive techniques such as endoscopy, colonoscopy along with biopsies are the current gold standard through which intestinal mucosa is assessed (Tooley, Howarth and Butler 2009). Whilst important, these techniques inform subjectively of the state of the mucosa and only of the tissue biopsied and offer little functional assessment of mucosal integrity. Currently, there are no diagnostic tests for stressed mucosa in a range of gastrointestinal illnesses such as IBS and other illness where mucosal stress may be a precursor to disease initiation (Hungin, Molloy- Bland, Claes *et al.* 2014). Diagnosis of IBS for example is made primarily using the Rome criteria. Little is known of the relationship between certain non-invasive markers of mucosal stress, (intestinal permeability and inflammation), intestinal gases and the microbiome during different illnesses. Non-invasive indicators of mucosal stress are therefore sought to help fill the diagnostic divide between assessment of FGID such as IBS and assessment of organic disease (Däbritz, Musci and Foell 2014).

6.6.2 Methods for assessing intestinal permeability

Currently, there exists a limited selection of permeability tests that can assess mucosal permeability in different geographic regions of the GIT (Tooley, Howarth and Butler 2009). Increased intestinal permeability indicates that the mucosal barrier is potentially compromised which may be an indicator of disease susceptibility. Radiolabelled/radioactive probes such as ^{51}Chromium Ethylene diamine tetra acetic acid is used to assess permeability, but due to the radioactivity is mostly confined to use in animal models (Butler 2008). Another method of

testing permeability across an epithelial membrane is the Ussing chamber. This *in vitro* technique is highly sensitive and useful for animal studies but is increasingly difficult to perform in human cohorts, as multiple biopsies are needed from different areas of the intestinal tract (Camilleri and Gorman 2007).

Non-invasive tests are highly desirable, relatively easy to collect, require minimal preparation and decrease the amount of patient stress. Furthermore, they allow real time data to be collected that does not alter the gastrointestinal environment. The dual sugar test is one such test that has been used successfully to assess mucosal repair in different clinical populations. In a study investigating mucosal repair in a cohort of coeliac patients the lactulose/rhamnose ratio test was a good indicator of improvement of the mucosal barrier in coeliac patients, after a diet free from gluten was implemented, indicating adherence to the diet (Cummins, Thompson, Butler *et al.* 2001). The dual sugar permeability test utilizes attributes of the mucosa, the transcellular and paracellular routes of absorption of the test sugars, which are then indicative of impaired control of the mucosal barrier. In paediatric populations with non-alcoholic fatty liver disease intestinal permeability was significantly higher when compared to healthy controls using the dual sugar test. Furthermore, there was an association with increased levels of intestinal permeability and symptom severity (Giorgio, Miele, Principessa *et al.* 2014). The combination of the dual sugar test with other non-invasive techniques to quantify specific bacterial levels in stool (PCR), may offer a unique approach to non-invasively evaluate mucosal stress in a clinical setting.

6.6.3 Faecal calprotectin as a marker of intestinal inflammation

Calprotectin is a 36 kDa cytosolic calcium binding protein predominately found in high levels in neutrophils (up to 60%) and to a lesser extent in monocytes and macrophages. Faecal calprotectin is remarkably stable and readily detected in faeces (Gisbert and Mcnicholl 2009), making it an excellent non-invasive marker of intestinal inflammation (Däbritz, Musci and Foell 2014). Clinically faecal calprotectin is used to assess disease activity in a variety of inflammatory GIT diseases (Waugh, Cummins, Royle *et al.* 2013). Normalisation of faecal calprotectin levels in IBD patients can be used to monitor disease activity in these patients and may also be a determinant of mucosal healing in IBD patients (Costa, Mumolo, Ceccarelli *et al.* 2005). Low-grade mucosal inflammation is increasingly recognised as a potential aetiological factor in certain subtypes of IBS (Ford and Talley 2011). Markers of intestinal inflammation that can be associated with specific bacteria (both beneficial and deleterious) or intestinal gases may have diagnostic potential in specific subtypes of IBS and other gastrointestinal diseases where mucosal stress is suspected.

6.7 Hypothetical use of an *E. coli*: beneficial microorganism index in faecal samples.

There is an unmet need to provide functional non-invasive diagnostic biomarkers to better understand the mechanisms of a range of diseases of the GIT. This thesis has endeavoured to investigate the relationship between an altered microbiome in diseases and disorders of differing severity as a first step to finding simple ways to both diagnose and monitor disease progression. One outcome of this thesis is the observation that *E. coli* is elevated in acutely damaged mucosa and that this is less evident in a more chronic, lower grade and episodic disorder (IBS). The studies in the animal model showed the highest levels of *E. coli* and that these were higher when histological damage was at its peak. In viral infections of childhood,

E. coli was elevated compared to controls but of a lower magnitude than that seen with the more florid inflammation after chemotherapy in a tumour-bearing animal model. In contrast the *E. coli* levels in IBS were similar to those seen in healthy children and the lowest seen in the disease states investigated in this thesis possibly reflecting the much lower extent of mucosal damage and inflammation in IBS. Whilst these levels alone might be useful in order to non-invasively discriminate the degree of mucosal stress the ratio of EC:BF was calculated (Table 6.1) in each setting.

In the viral infection setting it was clear that the ratio of EC:BF ratio showed a significant difference in RV, where the ratio was similar to healthy controls and significantly lower than the HBVo2 group ($p=0.05$). The RV levels were significantly higher to those seen in IBS ($p=0.003$). This is preliminary evidence that an EC:BF ratio may provide a simple paradigm for monitoring the relationship of an altered microbiome with the induction of mucosal stress and damage (Table 6.1). This could be further investigated in relation to both mucosal immune dysfunction and in certain settings of systematic immune dysregulation.

Table 6-1The *E. coli:Bifidobacterium* spp. ratio in chemotherapy-induced mucositis, two viral infections and IBS patients. EC, *E. coli*, BF, *Bifidobacterium* spp., RV, rotavirus, HBoV2, human boca virus 2. IBS, irritable bowel syndrome.

Illness	EC:BF (mean ± SEM)
EC:BF Caecal 72-96h mucositis	73.06 ± 34.65
EC:BF Caecal controls mucositis	0.00175 ± 0.001
EC:BF Colon 72-96h mucositis	14.44 ± 8.91
EC:BF Colon controls mucositis	0.00175 ± 0.001
EC:BF HBoV2	38.59 ± 32.50
EC:BF RV	1.954 ± 0.77
EC:BF Healthy controls (virus study)	3.180 ± 1.83
EC:BF IBS	1.479 ± 0.07

6.8 Future interventions

Illnesses of the gastrointestinal tract are increasingly recognized as not just of organic origin, but rather a combination of organic and functional aspects of microbial output and compositional changes in the intestinal microbiome. Currently, there is little data published on the intestinal microbiome in healthy humans, and the mechanistic role it plays in health. There is however, a large amount of published data from animal models linking intestinal microorganisms with certain structural changes in the mucosa that are related to health, such as decreasing intestinal permeability and inflammation. This now needs to be carried out systematically in the human arena. Non-invasive testing offers unique possibilities to monitor a range of metabolic changes in the GIT, before, during and after treatment. As yet, there is no clear definition of what constitutes a healthy intestinal microbiome. However, there is emerging evidence that shows compositional differences in specific microbial populations within the intestinal microbiome in specific gastrointestinal illnesses compared with people that are presumably healthy, or at least free from gastrointestinal illness at time of sampling. The intestinal microbiome and the mucosal interface are now at the forefront of an emerging new paradigm that recognises the way illnesses both intra- and extra-intestinally develop and are initiated.

Studies should now be undertaken in which a comprehensive inventory of the intestinal microbiome is taken at the start of a study and monitored at regular intervals throughout the treatment regime. Evaluation of intestinal gases that can be monitored non-invasively and related to specific bacteria or bacterial activity in the microbiome needs to be undertaken. This may give a clearer understanding of microbial activity throughout clinical management

involving pharmaceutical treatment regimens and/or for monitoring the effect of different dietary patterns.

The effect of probiotic administration on the endogenous microbiome needs to be elucidated, as this will provide evidence for formulating these interventions into the future. Indeed, the effectiveness of probiotic administration needs to acknowledge the high degree of variability across human populations and that one probiotic may not be suited to individuals with the same illness. This may account for the highly variable outcomes of RCTs investigating probiotics, as they tend to use the same probiotic for many illnesses and conditions.

Finally, endogenous beneficial microorganisms offer a vast reservoir of potential therapeutic options. The ability to select endogenous strains specific for an individual's microbial profile is an exciting prospect for the development of personalized medicine. This now needs to be rigorously examined in randomised controlled trials in human populations. Indeed, as this thesis has shown, certain endogenous microorganisms of the microbiome and their metabolic activity may promote barrier function, decrease intestinal inflammation and reduce diarrhoea.

6.9 Future directions

The intestinal microbiome is composed of trillions of microorganisms with a balanced, correctly functioning microbiome positively associated with human health. More importantly however is the increased awareness that an altered microbiome can disrupt the mucosal barrier leading to mucosal stress triggering immune system dysregulation leading to pathology. This is clearly illustrated with an increased susceptibility to GIT problems such as post-infectious IBS and the occurrence of *C. difficile* infection after antibiotic treatment. Overall it is clear that the stress induced by chemotherapeutic drugs, viral infections and a syndrome of unknown aetiology can markedly modify the endogenous microbiome and the relative levels of potentially protective microorganisms to those that may be deleterious.

The important questions are,

1.) Will a particular signature of an individual's microbiome constitute a protective element to the likelihood and progression of pathology?

2.) Could this aid in the diagnosis and selective design of preventative treatment regimens?

3.) Will selective augmentation of particular microorganisms e.g. probiotics that promote endogenous beneficial microorganisms and in turn diminish the colonization and activity of putative pathogens such as *E. coli*, or SRB perceptibly alter the course of illness?

4.) Will development of non-invasive methods to monitor and identify a stressed mucosa aid in understanding the mechanisms of microbiome and host interactions in GIT disease?

Several aspects of this thesis in part begin to answer these questions. It is now important to build on some of these associations derived from retrospective studies and to translate these

to prospective clinical trials utilizing the non-invasive tools described here and newer functional techniques as they become available.

New non-invasive biomarkers specifically targeting the intestinal mucosa have the ability to provide real time information as to the state of the microbiome and the mucosa during certain treatments such as chemotherapy-induced mucositis, or as an adjunct to the management of certain illnesses such as acute gastroenteritis and IBS. If mucosal stress is a precursor to illness, as others have postulated, then markers of mucosal health may be predictive of disease susceptibility and help in the understanding of lifestyle and environmental prevention strategies. Further research is needed into non-invasive markers of mucosal stress, which will help identify a stressed mucosa and enable more targeted therapeutic approaches.

6.10 Conclusions

To conclude, the aims of this thesis have broadly been met with some significant associations observed between selected endogenous microorganisms of the intestinal microbiome, clinical symptoms of stressed mucosa and non-invasive measurements of mucosal stress in three disparate settings. This has been shown in an animal model and two human studies, where levels of beneficial endogenous microorganisms and their metabolic activity appear to be protective against adverse changes in the GIT environment. This thesis describes selected changes in the microbiome during stress events, providing some scientific evidence to support the design of future intervention studies in a range of clinical settings. This may involve selectively augmenting the endogenous microbiome with either probiotics, prebiotics or synbiotics to promote not only mucosal health, but also the maintenance and restoration of the mucosal barrier under stress.

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